UNIVERSIDADE FEDERAL DE UBERLÂNDIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE
FACULDADE DE MEDICINA

ANÁLISE DE BIOMARCADORES SALIVARES POR ESPECTROSCOPIA ATR-FTIR E EXPRESSÃO DE MICRORNAS PLASMÁTICOS EM INDIVÍDUOS TREINADOS FRENTE A DIFERENTES PROTOCOLOS DE EXERCÍCIO FÍSICO

ADRIELE VIEIRA DE SOUZA

DOUTORADO EM CIÊNCIAS DA SAÚDE

2023

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Tese apresentada ao Programa de Pós-Graduação da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Doutora em Ciências da Saúde.

Área de concentração: Ciências da saúde.

Orientador: Prof. Dr. Foued Salmen Espindola.

UBERLÂNDIA – MG

2023

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S729a 2023

Souza, Adriele Vieira de, 1993-

Análise de biomarcadores salivares por espectroscopia ATR-FTIR e expressão de micrornas plasmáticos em indivíduos treinados frente a diferentes protocolos de exercício físico [recurso eletrônico] / Adriele Vieira de Souza. - 2025.

Orientador: Foued Salmen Espindola.

Tese (Doutorado) - Universidade Federal de Uberlândia, Programa de Programa de Pós-graduação em Ciências da Saúde.

Modo de acesso: Internet.

Disponível em: http://doi.org/10.14393/ufu.te.2025.5062

Inclui bibliografia.

Inclui ilustrações.

1. Ciências médicas. I. Espindola, Foued Salmen, 1957-, (Orient.). II. Universidade Federal de Uberlândia. Programa de Programa de Pós-graduação em Ciências da Saúde. III. Título.

CDU: 61

Nelson Marcos Ferreira
Bibliotecário-Documentalista - CRB-6/3074

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Programa de Pós-Graduação em:	Ciências da Saúde				
Defesa de:	Tese de Doutorado Nº 03/PPCSA				
Data:	25.04.2023	Hora de início:	09:20h	Hora de encerramento:	13:00h
Matrícula do Discente:	11813CSD001				
Nome do Discente:	Adriele Vieira de Souza				
Título do Trabalho:	"Análise de biomarcadores salivares por espectroscopia ATR-FTIR e expressão de microRNAs plasmáticos em indivíduos treinados frente a diferentes protocolos de exercício físico."				
Área de concentração:	Ciências da Saúde				
Linha de pesquisa:	3:Fisiopatologia das Doenças e Agravos à Saúde				
Projeto de Pesquisa de vinculação:	Desenvolvimento E Caracterização De Complexos Bioativos Nanoestruturados				

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Referência: Processo nº 23117.028925/2023-16 SEI nº 4448167

FOLHA DE APROVAÇÃO

Adriele Vieira de Souza.

Análise de biomarcadores salivares por espectroscopia ATR-FTIR e expressão de microRNAs plasmáticos em indivíduos treinados frente a diferentes protocolos de exercício físico.

Presidente da banca: Prof. Dr. Foued Salmen Espindola

Tese apresentada ao Programa de Pós-Graduação da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Doutora em Ciências da Saúde.

Área de concentração: Ciências da saúde.

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DEDICATÓRIA

Aos meus pais, Adriana e João, pelo amor incondicional, carinho, apoio e incentivo.

AGRADECIMENTOS

Aos meus pais João e Adriana, pelo amor, apoio e incentivo, em tudo o que faço. Obrigada por tudo o que me ensinaram durante todos estes anos, amo muito vocês.

À toda a minha família, que sempre me apoiou. Agradecimentos especiais ao meu irmão Hugo, avó Zenaide, cunhada Sabrina e primas Kymberlly e Larissa, que amo tanto e sempre estão presentes em minha vida. E claro, ao meu sobrinho Caleb, que é o neném mais lindo desse mundo.

Ao meu orientador, Prof. Dr. Foued, que é um homem e profissional incrível, que eu admiro muito. Obrigada não só por esse, mas por todos os outros trabalhos e pesquisas que foram desenvolvidas ao longo dos anos de Labibi. Obrigada pela disponibilidade, ensinamentos e compreensão durante todo este tempo.

À minha co-orientadora, Profª. Drª. Renata Roland, por toda a dedicação e amizade que tem comigo durante todos estes anos de laboratório.

Ao meu amigo Douglas Caixeta, que me ajudou muito no desenvolvimento desta pesquisa e de tantas outras. E pela amizade de milhões, te amo.

A todos os meus amigos companheiros de laboratório, especialmente Danielle Diniz, Poliana Ribeiro, Heitor Cappato, Camila Natalli, Leonardo Gomes, Mariane Muraoka, Letícia Afonso, Einy Siqueira, Pedro Henrique, Nathalia, André, Fábio, Júlia, Jéssica Giolo, Ana e Vinícius.

Aos professores Guilherme Puga, Robinson Sabino e Yara Maia, pela disponibilidade e colaboração nas pesquisas.

Aos meus amigos Jessica Souza, Adelino Azambuja, Aline Cunha, Helio Juliano, Denise Fernandes, Luiza Diniz, Paulo Cuevas, Lorena Polloni, Lívia Martins, Marina Negreto, Ana Paula Oliveira, Mariana Nunes, Alessandra Pissolati e Pedro Antunes. Esses, juntamente com outros amigos de laboratório, são pessoas

muito especiais pra mim. Muitos aqui nem fazem ideia da importância do apoio que me deram para que eu finalizasse essa etapa. Amo vocês.

A todos os voluntários que deram o seu suor para essa pesquisa (literalmente), muito obrigado! Sei que não foi fácil o grande número de visitas, os exercícios exaustivos, quantidade de coletas e ainda o grande tempo de espera pós-exercício.

À Universidade Federal de Uberlândia e aos órgãos de fomento FAPEMIG, CNPq e Capes, por toda a estrutura e apoio financeiro fornecidos.

"No matter how dark the night, morning always comes, and our journey begins anew"

(Final Fantasy X)

RESUMO

Introdução: A análise das alterações nos diferentes fluidos biológicos promovidas pelo exercício pode levar à descoberta de novos biomarcadores, capazes de melhorar a compreensão dos mecanismos relativos ao estado de treinamento e desempenho de atletas. Nesse sentido, a técnica de Espectroscopia no Infravermelho por Transformada de Fourier (FTIR) tem sido amplamente aplicada para a determinação de novos marcadores salivares, uma vez que este método analítico permite a caracterização de diferentes moléculas com alta sensibilidade e custo benefício. Além disso, estudos moleculares envolvendo a análise de microRNAs (miRNAs) (pequenas moléculas de RNA não codificantes envolvidas no processo de regulação pós-transcricional da expressão gênica), tem ganhado destaque na medicina esportiva, uma vez que seus níveis plasmáticos podem mudar em resposta ao exercício agudo e ao treinamento, de modo que essas moléculas são classificadas como mediadoras de respostas adaptativas ao exercício. No entanto, não está claro como os níveis destes marcadores variam de acordo com o tipo, duração e intensidade do exercício. **Objetivo:** determinar biomarcadores através da análise de alterações nos modos vibracionais de amostras salivares e expressão de miRNAs plasmáticos em indivíduos treinados frente a diferentes protocolos de exercício físico. **Material e métodos:** amostras de saliva e sangue foram coletadas de indivíduos do sexo masculino nos momentos: pré-exercício, pós-exercício e 3 horas pós-exercício. Os protocolos incluíram sessões agudas de exercício contínuo (EC), exercício intervalado de alta intensidade (HIIE) e exercício resistido (ER). As amostras salivares foram analisadas por ATR-FTIR e a técnica de RT-qPCR foi usada para determinar a expressão de diferentes miRNAs. **Resultados:** observou-se através da análise de ATR-FTIR, que os componentes bioquímicos salivares foram alterados conforme o protocolo de exercício realizado, sugerindo alguns picos espectrais específicos como biomarcadores dos mesmos. O EC apresentou um padrão de espectro salivar

semelhante ao HIIE, enquanto que o ER apresentou pequenas alterações. Também foi desenvolvido um algoritmo capaz de determinar uma faixa de espectro relacionada à determinação de intensidade do exercício. Em relação a expressão dos diferentes miRNAs analisados, foi verificado um aumento nos níveis de miR-23a com o EC. **Conclusão:** pelo nosso conhecimento, o presente trabalho é o primeiro a avaliar a expressão de miR-23a em amostras plasmáticas após exercício contínuo, contribuindo para uma melhor compreensão desta modalidade em relação a miRNAs circulantes e possíveis efeitos sobre as vias mitocondriais, uma vez que este miRNA tem como alvo o coativador 1-alfa do receptor gama ativado por proliferador de peroxissomo (PGC-1α). Em relação aos resultados obtidos com o FTIR, este é o primeiro estudo que comparou mudanças nos espectros salivares após diferentes protocolos de exercício, sugerindo picos de espectro como biomarcadores e o primeiro a desenvolver o referido algoritmo. Destacamos então, a espectroscopia por infravermelho como uma ferramenta útil, rápida e eficiente para avaliar as alterações metabólicas promovidas pelo exercício, permitindo o uso da saliva como uma alternativa custo-efetiva para o estudo do estado de treinamento e desempenho de atletas.

Palavras-chave: biomarcador salivar, FTIR, HIIT, epigenética, miRNAs circulantes.

ABSTRACT

Introduction: Analysis of the alterations in the different biological fluids induced by exercise can lead to the discovery of new biomarkers, that can enhance our understanding of the mechanisms related to the training status and performance of athletes. In this way, the Fourier Transform Infrared Spectroscopy (FTIR) technique has been widely applied for the determination of new salivary markers, since this analytical method allows the characterization of different molecules with high sensitivity and cost-effectiveness. In addition, molecular studies involving the analysis of microRNAs (miRNAs) (small non-coding RNA molecules involved in the process of post-transcriptional regulation of gene expression), have gained prominence in sports medicine, since their plasmatic levels can change in response to acute exercise and training, making these molecules classified as mediators of adaptive exercise responses. However, it is not clear how the levels of these markers change according to the type, duration and intensity of exercise. **Objective:** To determine biomarkers through the analysis of alterations in the vibrational modes of salivary samples and expression of plasmatic miRNAs in trained individuals following different protocols of physical exercise. **Material and methods:** Saliva and blood samples were collected from male individuals at the moments: pre-exercise, post-exercise and 3 hours post-exercise. Protocols included acute bouts of continuous exercise (CE), high-intensity interval exercise (HIIE), and resistance exercise (RE). Salivary samples were analyzed by ATR-FTIR and the RT-qPCR technique was used to determine the expression of different miRNAs. **Results:** It was observed through the ATR-FTIR analysis that the salivary biochemical components were altered according to the exercise protocol performed, suggesting some specific spectral peaks as their biomarkers. CE showed a salivary spectrum pattern similar to HIIE, while RE showed minor changes. An algorithm capable of determining a spectrum range related to the

determination of exercise intensity was also developed. Regarding the expression of the different analyzed miRNAs, an increase in miR-23a levels was observed following CE. **Conclusion:** to our knowledge, the present work is the first to evaluate miR-23a expression in plasma samples after continuous exercise, contributing to a better understanding of this modality in relation to circulating miRNAs and possible effects on mitochondrial pathways, since this miRNA targets peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Regarding the results obtained with FTIR, this is the first study that compared changes in salivary spectra after different exercise protocols, suggesting spectrum peaks as biomarkers and the first to develop the referred algorithm. Therefore, we highlight infrared spectroscopy as a useful, fast and efficient tool to evaluate the metabolic alterations promoted by exercise, allowing the use of saliva as a cost-effective alternative for studying the state of training and performance of athletes.

Keywords: salivary biomarker, FTIR, HIIT, epigenetics, circulating miRNAs.

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LISTA DE ABREVIATURAS E SIGLAS

12-RM	*12-Repetition Maximum Test*
3h post-ex	*3 hours post-exercise*
ANOVA-RM	*Analysis of Variance with Repeated Measures*
ATR	Reflexão Total Atenuada/ *Attenuated Total Reflection*
BMI	*Body Mass Index*
cDNA	*Complementary Deoxyribonucleic Acid*
Cel-miR-39-3p	*Caenorhabditis elegans miRNA-39-3p*
DHEA	Desidroepiandrosterona
EC/CE	Exercício contínuo/ *Continuous exercise*
ER/RE	Exercício resistido/ *Resistance exercise*
FCG	Fluido Crevicular Gengival
FTIR	Espectroscopia no infravermelho por transformada de Fourier/ *Fourier Transform Infrared Spectroscopy*
HIIE	Exercício Intervalado de Alta Intensidade/ *High-Intensity Interval Exercise*
HIIT	*High-Intensity Interval Training*
IR	Infravermelho médio
miRNAs	microRNAs
mRNA	RNA mensageiro
PGC-1α	Coativador 1-alfa do receptor gama ativado por proliferador de peroxissomo/*Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha*
Post-ex	*Post-exercise*
Pre-ex	*Pre-exercise*
RISC	Complexo de silenciamento induzido por RNA
RNA	Ácido ribonucleico/ *Ribonucleic Acid*
RT-qPCR	*Quantitative Reverse Transcription Polymerase Chain Reaction*
SCN^-	*Thiocyanates*

SUMÁRIO

1. INTRODUÇÃO

A prática de exercícios físicos traz inúmeros benefícios para a saúde, podendo aumentar a expectativa de vida e reduzir os riscos de doenças como diabetes, hipertensão, câncer e doenças cardiovasculares. Os diferentes efeitos do exercício promovidos à saúde devem-se a ativação de mecanismos moleculares e vias bioquímicas que geram respostas fisiológicas que podem variar conforme a prática de distintas modalidades. O exercício resistido, por exemplo, apresenta um efeito notável no aumento da massa e força muscular, além de aumentar a sensibilidade à insulina e a oxidação da glicose. Enquanto que o exercício aeróbico demonstra efeito pronunciado na melhora da aptidão cardiorrespiratória, nos níveis de colesterol de lipoproteína de alta densidade, triglicerídeos, glicemia de jejum e pressão arterial (HAWLEY, 2004; PINCKARD; BASKIN; STANFORD, 2019; WARBURTON; NICOL; BREDIN, 2006; WEN; WAI; TSAI; YANG *et al.*, 2011).

Evidências sugerem que o treino intervalado de alta intensidade (HIIT – do inglês "high-intensity interval training"), caracterizado pela prática de exercícios que intercalam ciclos de explosão e recuperação, pode ser uma alternativa viável ao treinamento aeróbico tradicional, uma vez que pode ser comparável ou até mesmo mais eficaz do que o exercício contínuo para melhorar diferentes parâmetros relacionados à capacidade aeróbica, aptidão anaeróbica, além de melhoras consideráveis em relação ao sistema vascular (GIBALA; LITTLE; MACDONALD; HAWLEY, 2012; RAMÍREZ-VÉLEZ; HERNÁNDEZ-QUIÑONES; TORDECILLA-SANDERS; ÁLVAREZ *et al.*, 2019; ZIEMANN; GRZYWACZ; LUSZCZYK; LASKOWSKI *et al.*, 2011).

O adequado monitoramento das alterações bioquímicas que ocorrem frente a diferentes estressores psicofisiológicos é de grande importância na medicina esportiva e do exercício. A análise das alterações nos diferentes fluidos biológicos

promovidas pelo exercício pode levar à descoberta de novos biomarcadores, capazes de determinar a intensidade do exercício, recuperação, lesão e, assim, melhorar a compreensão dos mecanismos relativos ao estado de treinamento e desempenho do atleta (LEE; FRAGALA; KAVOURAS; QUEEN *et al.*, 2017; PALACIOS; PEDRERO-CHAMIZO; PALACIOS; MAROTO-SÁNCHEZ *et al.*, 2015).

Nesse contexto, tem crescido o interesse na busca por biomarcadores salivares. A saliva é um fluido complexo composto de água e uma grande variedade de substâncias inorgânicas e orgânicas, como eletrólitos, muco, enzimas e compostos antibacterianos. Este biofluido tem sido amplamente utilizado em diagnósticos clínicos e pesquisas, uma vez que apresenta diversas vantagens em relação a outros fluidos, como o plasma. A coleta de saliva é não invasiva em comparação com a flebotomia, não causa dor ou desconforto e é menos propensa a causar estresse aos indivíduos (MALAMUD, 2011; TIWARI, 2011).

Dentre os diversos biomarcadores salivares relevantes estudados no exercício físico, podemos citar o lactato salivar, atividade amilase, óxido nítrico, proteína total, marcadores de estresse oxidativo e hormônios como testosterona e cortisol (DE OLIVEIRA; BESSA; LAMOUNIER; DE SANTANA *et al.*, 2010; DIAZ; BOCANEGRA; TEIXEIRA; SOARES *et al.*, 2013; HAYES; GRACE; BAKER; SCULTHORPE, 2015; PALACIOS; PEDRERO-CHAMIZO; PALACIOS; MAROTO-SÁNCHEZ *et al.*, 2015; SOUZA; GIOLO; TEIXEIRA; VILELA *et al.*, 2019).

A busca por análises mais simples e econômicas para a determinação dos componentes salivares torna ferramentas como a espectroscopia no infravermelho por transformada de Fourier com reflectância total atenuada (ATR-FTIR) uma interessante escolha analítica. Por meio da análise de FTIR, é possível caracterizar efetivamente as moléculas biológicas em fluidos, como proteínas, açúcares e

lipídios, com alta sensibilidade e sem o uso de reagentes. Além disso, esta ferramenta requer pequenas quantidades de amostra (muitas vezes um fator limitante nos estudos), permitindo obter múltiplas determinações moleculares com uma quantidade mínima de material (THEAKSTONE; RINALDI; BUTLER; CAMERON *et al.*, 2021).

Além do monitoramento das alterações bioquímicas, é de grande importância na medicina esportiva o estudo dos mecanismos moleculares genéticos envolvidos na regulação das diferentes alterações inerentes à adaptação ao exercício. Nesse sentido, estudos têm demonstrado que diversos processos como angiogênese, inflamação, metabolismo mitocondrial e hipertrofia do músculo esquelético, podem ser regulados por meio de microRNAs (miRNAs) (CHAN; ZHANG; HEMANN; MAHONEY *et al.*, 2009; DAVIDSEN; GALLAGHER; HARTMAN; TARNOPOLSKY *et al.*, 2011; DAVIDSON-MONCADA; PAPAVASILIOU; TAM, 2010; FERNANDES; BARAUNA; NEGRAO; PHILLIPS *et al.*, 2015). Essas moléculas consistem em pequenos RNAs não codificantes (geralmente de 19 a 24 nucleotídeos de comprimento) que desempenham um papel importante na regulação pós-transcricional da expressão gênica, promovendo a degradação do RNA mensageiro (mRNA) ou a inibição da tradução de proteínas. Sob estímulos fisiológicos como exercício físico ou processos patológicos, essas moléculas podem ser liberadas de diferentes tipos de células e tecidos para a circulação, via vesículas ou ligadas a proteínas. Esses miRNAs circulantes podem então ser transportados para as células-alvo e regular positivamente ou negativamente genes importantes envolvidos em diferentes processos fisiológicos (CONDRAT; THOMPSON; BARBU; BUGNAR *et al.*, 2020; ETHERIDGE; LEE; HOOD; GALAS *et al.*, 2011; MOHR; MOTT, 2015).

Estudos mostram que diferentes tipos, intensidade, frequência e duração do exercício podem levar a alterações bioquímicas distintas e, portanto, interferir de forma diferenciada em importantes parâmetros fisiológicos relacionados à

adaptação do exercício (LEAF; KLEINMAN; HAMILTON; BARSTOW, 1997; MOGHARNASI; CHERAGH-BIRJANDI; CHERAGH-BIRJANDI; TAHERICHADORNESHIN, 2017; PATEL; ALKHAWAM; MADANIEH; SHAH *et al.*, 2017). Também se sabe que a expressão de determinados miRNAs pode ser alterada de acordo com o estado do treinamento, o tipo e a intensidade do exercício realizado (FARALDI; GOMARASCA; SANSONI; PEREGO *et al.*, 2019; FERNÁNDEZ-SANJURJO; ÚBEDA; FERNÁNDEZ-GARCÍA; DEL VALLE *et al.*, 2020; RAMOS; LO; ESTEPHAN; TAI *et al.*, 2018; UHLEMANN; MÖBIUS-WINKLER; FIKENZER; ADAM *et al.*, 2014).

Pelo nosso conhecimento, até o momento, não existem estudos que avaliem e comparem os espectros salivares de FTIR frente a diferentes protocolos de exercícios. Além disso, não está claro como os níveis de expressão de miRNAs plasmáticos se alteram de acordo com tipos específicos de intervenções. Assim, a elucidação da alteração diferencial destas moléculas é importante para o entendimento dos processos moleculares que levam aos efeitos adaptativos de cada protocolo de exercício físico, além de possibilitar o uso da saliva como uma ferramenta custo-efetiva em diferentes estudos na área de medicina esportiva.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Saliva e Exercício Físico

A saliva compreende um fluido transparente, composta de modo majoritário por água (cerca de 98-99%), além de diversas substâncias inorgânicas e orgânicas. Apresenta uma grande variedade de eletrólitos (tais como sódio, potássio, cálcio, cloreto, magnésio, bicarbonato e fosfato), além de diversas enzimas, hormônios, anticorpos, diferentes componentes antimicrobianos e fatores de crescimento. Outros componentes incluem variadas proteínas, polipeptídeos e oligopeptídeos, glicose e produtos nitrogenados (CHICHARRO; LUCIA; PEREZ; VAQUERO *et al.*, 1998; DE ALMEIDA PDEL; GRÉGIO; MACHADO; DE LIMA *et al.*, 2008; KAUFMAN; LAMSTER, 2002).

Este fluido apresenta diversas funções, tais como: a lubrificação dos tecidos duros e moles da cavidade bucal; facilitação da deglutição, mastigação e fonação; digestão (responsável pelas etapas iniciais da digestão dos carboidratos promovida pela ação da enzima alfa-amilase salivar); ação tamponante (responsável por manter o pH da saliva e promover processos de remineralizarão dental), ação antimicrobiana (devido a presença de componentes imunológicos como a imunoglobulina A e enzimas como lisozima, lactoferrina e peroxidases, além de proteínas como mucinas, glicoproteínas, histatinas e proteínas ricas em prolina); entre outras funções (CHICHARRO; LUCIA; PEREZ; VAQUERO *et al.*, 1998; DE ALMEIDA PDEL; GRÉGIO; MACHADO; DE LIMA *et al.*, 2008; HUMPHREY; WILLIAMSON, 2001; KAUFMAN; LAMSTER, 2002).

O processo de secreção da saliva na cavidade bucal envolve três pares de glândulas salivares maiores (parótida, submandibular e sublingual) e muitas glândulas salivares menores, distribuídas pela mucosa oral (CARPENTER, 2013). A saliva pode ser denominada como glândula-específica, quando coletada

diretamente de uma glândula, ou como saliva total, que representa uma mistura das secreções das glândulas salivares e outros componentes. O fluido mais amplamente estudado constitui a saliva total, com o intuito de elucidar tanto alterações locais quanto sistêmicas (MALAMUD, 2011; NAVAZESH, 1993). A saliva total compreende uma mistura de fluidos orais, incluindo secreções das glândulas salivares maiores e menores, além de vários outros constituintes como o fluido crevicular gengival (FCG), secreções brônquicas e nasais, além de derivados de sangue e soro, células epiteliais, produtos bacterianos e debris de alimentos (KAUFMAN; LAMSTER, 2002).

A coleta salivar apresenta diversas vantagens em relação a coleta de sangue, por ser um método não-invasivo, não exigir um profissional altamente treinado, não requerer equipamento específico para coleta, não causar dor ou desconforto, além de ser mais seguro e menos propenso a causar estresse aos indivíduos. Esta última vantagem torna-se muito importante ao avaliar certas populações específicas, como crianças, idosos e atletas, uma vez que a coleta de sangue pode ser inviabilizada nesses indivíduos devido a diferentes fatores, como medo, fragilidade, dificuldade técnica de coleta e competições. Além disso, a coleta de saliva constitui um método mais econômica, o que favorece a realização de pesquisas envolvendo populações maiores (KAUFMAN; LAMSTER, 2002; MALAMUD, 2011).

Em relação aos métodos de coleta, a saliva total pode ser obtida por métodos estimulantes ou não. A saliva estimulada pode ser coletada utilizando estímulos mecânicos (como por exemplo, através da mastigação de parafilme) ou por estímulos químicos/gustativos (como observado na utilização de ácido cítrico ou gomas de sabor). Já a saliva não estimulada pode ser obtida pelo método de expectoração ou método de cuspe, em que o indivíduo realiza a deposição do fluido salivar acumulado de forma passiva em um tubo de coleta durante um determinado período de tempo. Existem ainda diferentes instrumentos

disponíveis no mercado para a coleta de saliva, dos quais destaca-se o dispositivo de nome comercial “Salivette®”. Este compreende um rolo cilíndrico de superfície lisa, que apresenta um algodão em seu interior capaz de absorver a saliva. Posteriormente à coleta, o dispositivo é submetido ao processo de centrifugação para a recuperação e viabilização do uso da amostra. É importante salientar que o método escolhido pelo pesquisador deve ser criteriosamente avaliado conforme o objetivo do estudo, uma vez que cada tipo de coleta pode apresentar diferenças em relação ao fluxo salivar, pH e alterações de diversos componentes. (GOLATOWSKI; SALAZAR; DHOPLE; HAMMER *et al.*, 2013; NAVAZESH, 1993; RABE; GESELL SALAZAR; FUCHS; KOCHER *et al.*, 2018).

A saliva tem sido muito utilizada para o estudo de diferentes biomarcadores, para o diagnóstico de doenças e para o monitoramento de drogas. Algumas doenças podem afetar as glândulas salivares de forma direta ou indireta, podendo influenciar na quantidade de saliva produzida, assim como alterar a sua composição. Tal fato faz com que a saliva tenha ganhado destaque no diagnóstico de diversas doenças hereditárias, autoimunes (como a síndrome de Sjögren), na detecção de tumores malignos, doenças infecciosas, além de ser usada na análise endócrina através do monitoramento de hormônios (KAUFMAN; LAMSTER, 2002; MALAMUD, 2011).

Na área do exercício físico, as análises salivares também apresentam diversas aplicações. Seu potencial pode ser empregado ao estudo de biomarcadores envolvidos a estressores fisiológicos e psicológicos (como observado nos atletas em competições) ou em diferentes avaliações crônicas (através da verificação de biomarcadores relacionados à sobrecarga de treinamento) (LINDSAY; COSTELLO, 2017). Além disso, a saliva tem sido utilizada na determinação dos níveis de hidratação corporal total (WALSH; MONTAGUE; CALLOW; ROWLANDS, 2004).

O exercício é capaz de promover diversas alterações nos componentes salivares, como imunoglobulinas, hormônios, lactato, proteínas e eletrólitos. Um importante indicador do estado imunológico de atletas consiste na avaliação da concentração salivar de IgA. Alguns estudos demonstram os baixos níveis de IgA relacionados com aumento à susceptibilidade a infecções nos casos de atividade física extenuante (BLANNIN; ROBSON; WALSH; CLARK *et al.*, 1998; GLEESON; MCDONALD; PYNE; CRIPPS *et al.*, 1999).

Análises laboratoriais de esteroides salivares durante o exercício também têm sido frequentemente utilizadas, uma vez que tais substâncias podem ser avaliadas de forma confiável na saliva. Os hormônios mais comumente avaliados correspondem ao cortisol salivar, testosterona e DHEA (desidroepiandrosterona). A análise salivar hormonal pode ser usada como indicativa de estresse de treinamento, ou mesmo com objetivo de verificação de dopping em competições (GATTI; DE PALO, 2011).

Outros importantes biomarcadores avaliados na saliva correspondem ao lactato, atividade de amilase salivar e concentração de proteína total, que podem ser utilizados como biomarcadores de intensidade de exercício (CALVO; CHICHARRO; BANDRES; LUCIA *et al.*, 1997; CHICHARRO; LUCIA; PEREZ; VAQUERO *et al.*, 1998; DE OLIVEIRA; BESSA; LAMOUNIER; DE SANTANA *et al.*, 2010). Além disso, também pode ser realizado na saliva a dosagem de compostos do sistema antioxidante (SOUZA; GIOLO; TEIXEIRA; VILELA *et al.*, 2019) e de nitrito salivar (representativo dos níveis de óxido nítrico associados aos efeitos do exercício físico sobre a vasodilatação) (BRYAN, 2015).

As análises de biomarcadores salivares podem então permitir o fornecimento de dados de grande importância para treinadores e pesquisadores da medicina esportiva, auxiliando na elaboração de treinos mais adaptados às

condições dos atletas e contribuindo para a elucidação dos diversos processos bioquímicos relacionados a adaptação ao exercício.

2.2. Espectroscopia FTIR

A espectroscopia no infravermelho por transformada de Fourier (FTIR), consiste em uma técnica baseada na absorção da radiação eletromagnética com comprimentos de onda na região do infravermelho médio (IR) (4000–400 cm-1). Funciona através da incidência de luz de característica policromática sobre a amostra e subsequente mensuração da quantidade de luz absorvida. Tal absorção, é característica da natureza das ligações químicas presentes na amostra analisada. Estas informações são coletadas e uma equação matemática é utilizada para converter os dados brutos (interferograma) em um espectro interpretável (OJEDA; DITTRICH, 2012; YANG; YANG; KONG; DONG *et al.*, 2015).

Os componentes básicos e o funcionamento de um equipamento de FTIR é resumido e esquematizado na Figura 1. Inicialmente, a radiação de uma fonte policromática é direcionada ao interferômetro de Michelson. Neste, a radiação colide com um divisor de feixes (do inglês “beamsplitter”), que é então dividida em dois feixes. Um destes é refletido para um espelho fixo, enquanto o outro é refletido em um espelho móvel. Após este processo, o feixe se recombina e são produzidos novamente dois feixes, um que retorna a fonte de energia e outro que vai em direção a amostra e é mensurado pelo detector. Os dados são então acumulados como interferogramas no computador e em seguida são convertidos em absorção óptica infravermelha em função do número de onda (OJEDA; DITTRICH, 2012).

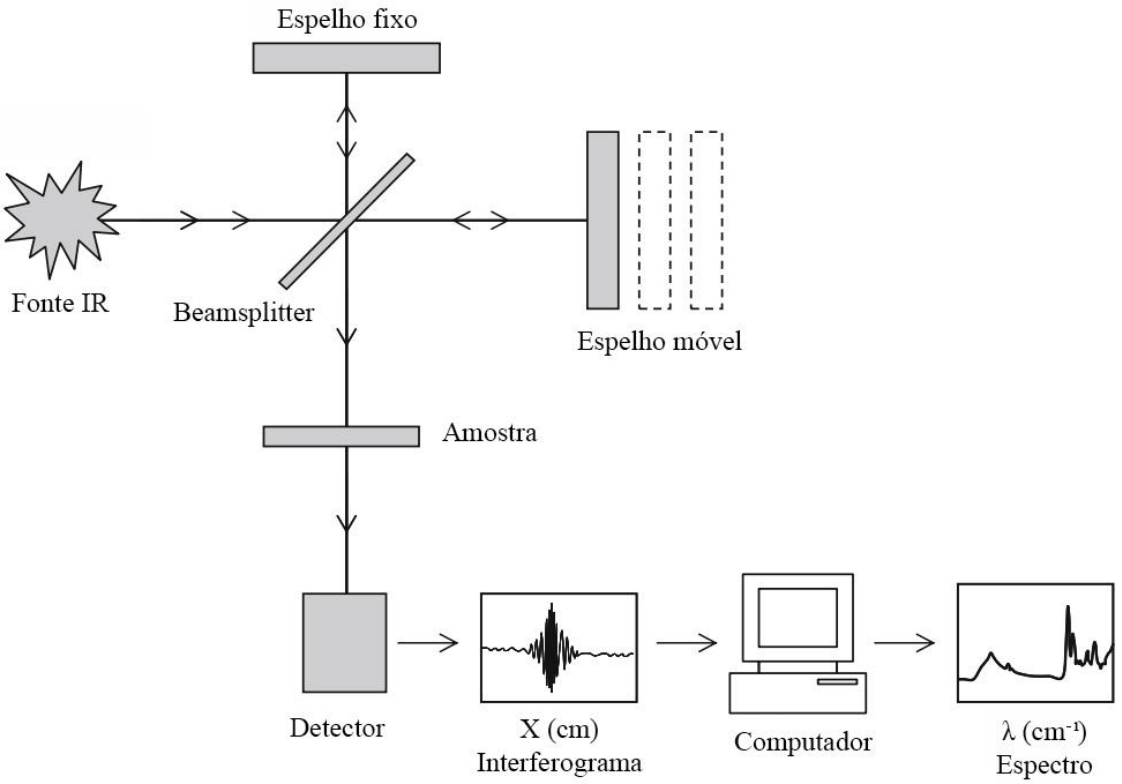

Figura 1. Componentes básicos e funcionamento de um aparelho de FTIR. Fonte IR: fonte de infravermelho médio; Beamsplitter, do inglês: divisor de feixes de luz. Fonte: Adaptado de Ojeda e Dittrich (2012).

A energia que é absorvida pela amostra pode ativar vibrações de ligações moleculares específicas, como de deformação axial (conhecido como *stretching,* que pode ser um estiramento do tipo simétrico ou assimétrico) e de deformação angular (conhecido como *bending*, podendo ser dos tipos tesoura, balanço, abano ou torção) (A SKOOG, 2000; OJEDA; DITTRICH, 2012). As deformações de estiramento envolvem a alteração do comprimento das ligações e estão representadas na Figura 2 (A-B), enquanto que as deformações de flexão envolvem alteração de seus ângulos e estão esquematizadas na Figura 3 (A-D).

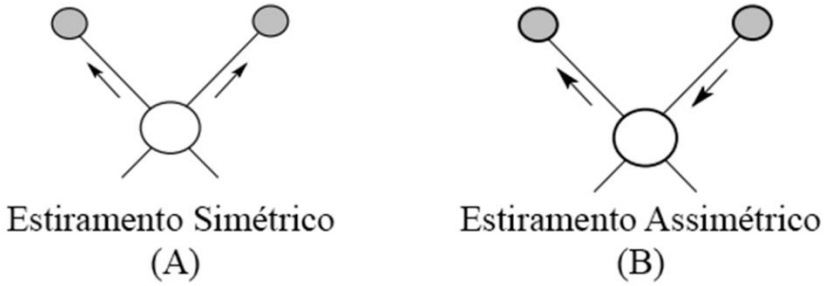

Figura 2. Tipos de vibrações moleculares do tipo S*tretching*. Em (A) tem-se o Estiramento Simétrico e em (B) o Estiramento Assimétrico. Fonte: Adaptado de Skoog et al. (2000).

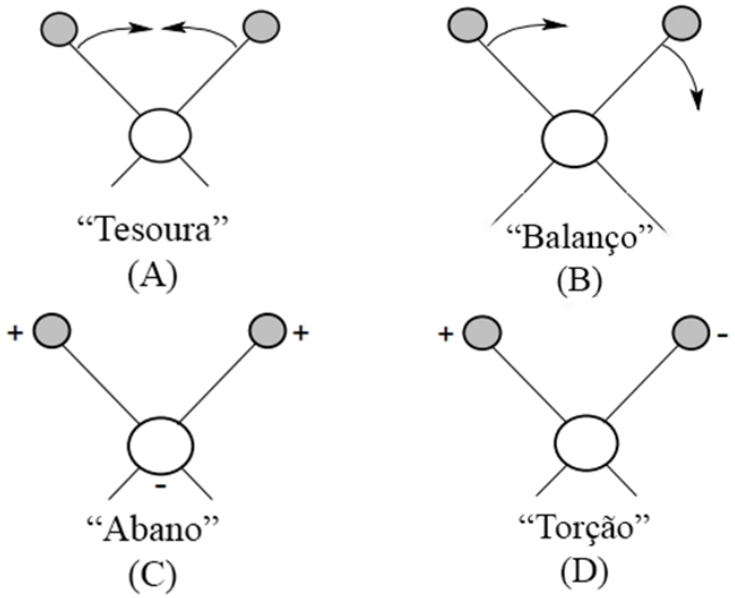

Figura 3. Tipos de vibrações moleculares do tipo *Bending. Em (A) tem-se o modo "Tesoura" ou "*scissoring", em (B) "Balanço" ou "rocking", em (C) "Abano" ou *"*wagging" e em (D) "Torção" ou *"*twisting". Os sinais de "+" e "-" indicam movimentos em direção ao plano e contra o plano, respectivamente. Fonte: Adaptado de SKOOG et al. (2000).

Cada composto apresenta um espectro infravermelho característico, que pode ser utilizado para a identificação e quantificação dos componentes da amostra. Assim, através desta análise é possível identificar variações nos grupos funcionais por meio da mensuração da vibração e rotação de moléculas influenciadas pelo IR, promovendo informações sobre a estrutura e composição química (tais como identificação de lipídios, proteínas, carboidratos e ácidos

nucléicos) referentes a amostras biológicas, como tecidos, células e fluidos corporais (FERREIRA; AGUIAR; SILVA; SANTOS *et al.*, 2020; YANG; YANG; KONG; DONG *et al.*, 2015).

Existem diferentes modos de análise de FTIR, tais como por transmissão, trasnflexão e reflexão total atenuada (ATR). Este último caracteriza-se pela passagem da luz infravermelha através de um cristal, de forma que a amostra é pressionada sobre ele. Tal método tem-se destacado por permitir a análise de espectros de forma altamente sensível, independentemente da espessura da amostra. Além disso, o modo ATR permite a análise de uma ampla gama amostral, incluindo sólidos e líquidos, sem a necessidade de etapas prévias de preparação ou diluição da amostra a ser analisada (GLASSFORD; BYRNE; KAZARIAN, 2013).

A metodologia de FTIR tem ganhado destaque como uma importante ferramenta em diversas áreas, por se tratar de um método econômico (uma vez que não utiliza reagentes), ser altamente sensível e viabilizar a realização de múltiplas determinações moleculares a partir de uma análise única da amostra (THEAKSTONE; RINALDI; BUTLER; CAMERON *et al.*, 2021). As aplicações do método incluem as ciências forenses, caracterização de compostos poliméricos, controle de qualidade e diagnóstico de doenças como câncer, diabetes e periodontite (CHÉRCOLES ASENSIO; SAN ANDRÉS MOYA; DE LA ROJA; GÓMEZ, 2009; MURO; DOTY; BUENO; HALÁMKOVÁ *et al.*, 2015; NOGUEIRA; BARRETO; FURUKAWA; ROVAI *et al.*, 2022; SALA; ANDERSON; BRENNAN; BUTLER *et al.*, 2020; SONG; CONG; WANG; ZHANG, 2020). As aplicações relacionadas a estudos de exercício físico são menos exploradas (CAETANO JÚNIOR; CARVALHO AGUIAR; FERREIRA-STRIXINO; JOSÉ RANIERO, 2017; CAETANO; STRIXINO; RANIERO, 2015; KHAUSTOVA; SHKURNIKOV; TONEVITSKY; ARTYUSHENKO *et al.*, 2010; VIEIRA; PUPIN; BHATTACHARJEE; SAKANE, 2021).

2.3. miRNAs e Exercício Físico

Os microRNAs (miRNAs) são pequenas moléculas de RNA não codificantes (19-24 nucleotídeos) que regulam a expressão gênica a nível pós-transcricional através da degradação do RNA mensageiro (mRNA) ou inibição da tradução de proteínas (FUKUNAGA, 2016; KOMATSU; KITAI; SUZUKI, 2023).

O processo de biogênese dos miRNAs tem início com a sua transcrição, que ocorre no núcleo pela ação da enzima RNA polimerase II, formando a molécula precursora definida como pri-miRNA. Posteriormente, o pri-miRNA é processado pela enzima Drosha, que promove a sua clivagem e formação da molécula de pre-miRNA, que é então transportada para a região do citoplasma. Em seguida, o pre-miRNA é clivado pela enzima Dicer, resultando na formação de um dúplex de RNA. Este é inserido ao complexo de silenciamento induzido por RNA (RISC), que promove a separação das fitas de RNA. A fita complementar sofre degradação, enquanto que a fita que permanece associada ao RISC constitui a molécula madura de miRNA (KOMATSU; KITAI; SUZUKI, 2023).

O processo de silenciamento ocorre devido à complementaridade de bases, em que o miRNA se liga ao mRNA alvo na região 3'UTR. Dependendo do grau dessa complementariedade, o RISC pode induzir a degradação do mRNA ou inibir a tradução do mRNA em proteínas (BARTEL, 2004). Tais processos envolvendo biogênese e funcionamento dos miRNAs não estão completamente elucidados, o que tem estimulado diversas pesquisas nessa área (KOMATSU; KITAI; SUZUKI, 2023).

Outra crescente área de estudo corresponde aos biomarcadores, em que os miRNAs circulantes são alvos promissores de investigação. Esses correspondem aos miRNAs que circulam pelo sangue e em outros fluidos corporais, como a saliva e o leite materno. Sabe-se que estes miRNAs são liberados por diferentes células e tecidos, podendo desempenhar um importante papel na comunicação

intercelular e na regulação de processos biológicos em tecidos distantes do local de origem. Além disso, certos estímulos fisiológicos, como exercícios físicos ou processos patológicos, podem estimular a liberação destas moléculas para a circulação, levando à uma regulação positiva ou negativa destes genes (CONDRAT; THOMPSON; BARBU; BUGNAR *et al.*, 2020; EL-DALY; GOUHAR; ABD ELMAGEED, 2023; ETHERIDGE; LEE; HOOD; GALAS *et al.*, 2011; MOHR; MOTT, 2015).

A identificação de miRNAs circulantes em resposta a uma determinada modalidade de exercício pode ser útil para o melhor entendimento dos processos moleculares que levam aos efeitos adaptativos de cada protocolo. Além de poder melhorar o monitoramento de respostas de desempenho dos atletas, detectar precocemente diferentes condições fisiopatológicas, viabilizar a prescrição individualizada de exercícios, analisar fatores de recuperação e evitar possíveis consequências indesejáveis advindas de um programa de treinamento (SILVA; IOP; ANDRADE; COSTA *et al.*, 2020).

São diversos os miRNAs envolvidos nos diferentes processos adaptativos promovidos pelo exercício físico, tais como o miR-133a, miR-126a, miR-146a, miR-221 e miR-23a. Estes estão relacionados com a regulação de vias referentes à hipertrofia, angiogênese, inflamação, metabolismo mitocondrial, entre outros (CHAN; ZHANG; HEMANN; MAHONEY *et al.*, 2009; DAVIDSEN; GALLAGHER; HARTMAN; TARNOPOLSKY *et al.*, 2011; DAVIDSON-MONCADA; PAPAVASILIOU; TAM, 2010; FERNANDES; BARAUNA; NEGRAO; PHILLIPS *et al.*, 2015).

O miR-133 é um dos mais explorados na área de exercício físico. Alguns estudos têm mostrado que ele apresenta papel fundamental na hipertrofia cardíaca (CARE; CATALUCCI; FELICETTI; BONCI *et al.*, 2007; WISLOFF; ELLINGSEN; KEMI, 2009), assim como o miR-1 (CARE; CATALUCCI; FELICETTI; BONCI *et al.*, 2007). Estes miRNAs são reguladores da hipertrofia

por silenciarem sequências de mRNA transcritas e, portanto, inibem a hipertrofia do coração. Assim, sua regulação negativa contribui para aumentar a taxa de tradução do mRNA e de síntese de proteínas (WISLOFF; ELLINGSEN; KEMI, 2009). Análises de bioinformática mostraram os possíveis alvos deste miRNA, que incluem: RhoA (proteína GTPase envolvida em processos celulares como o remodelamento do citoesqueleto, migração, adesão, endocitose e progressão do ciclo celular (O'CONNOR; CHEN, 2013); Cdc42 (proteína quinase envolvida em hipertrofia) e Nelf-A/WHSC2 (fator nuclear envolvido na cardiogênese) (CARE; CATALUCCI; FELICETTI; BONCI *et al.*, 2007).

Outros miRNAs, como o miR-221 e o miR-126, estão associados a processos de angiogênese (EBRAHIMI; RASTEGAR-MOGHADDAM; MOHAMMADIPOUR, 2023; KUEHBACHER; URBICH; ZEIHER; DIMMELER, 2007; POLISENO; TUCCOLI; MARIANI; EVANGELISTA *et al.*, 2006), enquanto que o miR-146 está mais associado a processos envolvendo inflamação (TAGANOV; BOLDIN; CHANG; BALTIMORE, 2006) e resposta celular a hipóxia (CHAN; ZHANG; HEMANN; MAHONEY *et al.*, 2009). Por último, tem-se o miR-23, que tem como alvo moléculas envolvidas na biogênese mitocondrial (JI; GOMEZ-CABRERA; VINA, 2006; LEWIS; BURGE; BARTEL, 2005).

3. OBJETIVOS

3.1. Objetivo geral

Avaliar os efeitos de diferentes sessões agudas de exercício físico (HIIE: exercício intervalado de alta intensidade; EC: exercício contínuo; e ER: exercício resistido) em indivíduos treinados sobre o perfil espectral salivar e análise da expressão de miRNAs plasmáticos.

3.2. Objetivos específicos

- Verificar se o perfil espectral salivar se altera diferencialmente de acordo com o exercício físico realizado (HIIE, CE, RE) através da técnica de ATR-FTIR;
- Definir biomarcadores salivares a partir dos picos vibracionais diferencialmente alterados;
- Desenvolver um algoritmo capaz de relacionar os valores do espectro com marcadores de intensidade de exercício;
- Comparar a expressão de miRNAs circulantes específicos, previamente relacionados a importantes modulações fisiológicas promovidas pelo exercício (miR-133a, miR-126a, miR-146a, miR-221 e miR-23a), em resposta a sessão aguda dos diferentes protocolos de exercício: CE, HIIIE e ER.

4. ARTIGOS CIENTÍFICOS

4.1 Artigo 1 – Título: *“‘Determination of salivary biomarkers using ATR-FTIR spectroscopy in different exercise protocols”*

Parecer do CEP consubstanciado (ANEXO A)

Normas de submissão para autores - Scientific Reports (ANEXO B)

Determination of salivary biomarkers using ATR-FTIR spectroscopy in different exercise protocols

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Abstract

The Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) has been applied for the determination of novel salivary biomarkers, since this analytical method allows the characterization of the biological molecules in this fluid with high sensitivity and in cost-effective way. In this work, we tested the hypothesis that the spectral profile of saliva presents distinct vibrational modes according to different exercises protocols and may be employed for exercise monitoring. For this, saliva samples were collected from thirteen trained male subjects at the moments: pre-exercise, post-exercise, and 3 hours post-exercise. Protocols included acute sessions of continuous exercise (CE), high-intensity interval exercise (HIIE) and resistance exercise (RE). It was seen through ATR-FTIR analysis, that the salivary biochemical components changed differently according to the exercise protocol performed, suggesting some specific spectral peaks as their biomarkers. CE showed a similar salivary spectrum pattern compared to HIIE, while the RE presented minor alterations. We also developed an algorithm capable of determining a spectrum range related to physical exercise intensity. This is the first study that compared changes in the saliva spectra following different exercise protocols and to suggest spectrum peaks as markers of specific types of exercises. We highlight the use of machine learning together with spectroscopy as a potential way to provide a useful, fast, and efficient tool to evaluate metabolic changes promoted by exercise, suggesting the use of saliva as a cost-effective alternative for the study of athlete training state and performance.

Keywords: FTIR, HIIT, salivary biomarker, exercise protocols.

Introduction

The adequate monitoring of the biochemical changes that occur in face of different psychophysiological stressors is of great importance in sports and exercise medicine. Analysis of changes in the different biological fluids promoted by exercise could lead to the discovery of novel biomarkers, capable of determining exercise intensity, recovery, injury, and then improve the understanding of mechanisms regarding athlete training status and performance [1,2].

In this context, interest has grown in the search for salivary biomarkers. Saliva is a complex fluid composed of water, and a large variety of inorganic and organic substances, such as electrolytes, mucus, enzymes and antibacterial compounds. This biofluid has been extensively used in clinical diagnosis and research, since it has several advantages over other fluids, such as plasma. Saliva collection is non-invasive compared with phlebotomy, does not cause pain or discomfort and is less likely to cause stress to the subjects [3,4]. Among the several relevant salivary biomarkers studied in physical exercise, we can cite the salivary lactate, amylase activity, nitric oxide, total protein, oxidative stress markers and hormones such as testosterone and cortisol [2,5-8].

The search for simpler and more cost-effective approaches for the determination of salivary components, makes tools such as Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) a great analytical choice. Through FTIR analysis, it is possible to effectively characterize the biological molecules in fluids, such as proteins, sugars, and lipids, with high sensitivity and without the use of reagents. Furthermore, this tool requires small amounts of sample (often a limiting factor in studies),

allowing to obtain multiple molecular determinations with a minimal amount of material.

Studies show that different types, intensity, frequency, and duration of exercise can lead do distinctive biochemical alterations, and therefore interfere differentially in important physiological parameters related do adaptation of exercise [9-11]. However, to our knowledge, there are no studies that evaluate and compare the salivary FTIR spectra following different types of exercises protocols.

In this work, we tested the hypothesis that the spectral profile of saliva is expressed differentially according to the physical exercise performed (high-intensity interval exercise, continuous exercise, and resistance exercise) so that the distinct vibrational modes can characterize these protocols and may be employed for exercise monitoring.

MATERIAL AND METHODS

Subjects

This study was performed with thirteen trained healthy males, who were nonsmoking, not taking regular/incidental medication or performance enhancing drugs. Subject characteristics are shown in Table 1.

Table 1. Subject Characteristics.

Age (years)	*27.62±1.28*
Height (cm)	*174.20±2.14*
Weight (Kg)	*72.07 ±1.77*
BMI (Kg/m^2)	*23.76± 0.54*
Body Fat (%)	*15.16±0.99*

BMI: Body Mass Index. Values presented as means ± SEM, n = 13.

All participants agreed and signed an informed consent form prior to admission to the study, and experiments were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Subjects reported being physically active, performing both aerobic and anaerobic physical exercise in the past 6 months at least 3 times a week. This study was approved by the Institutional Review Board of the Federal University of Uberlandia (nº. 1.908.151).

Exercise protocols

Aerobic capacity was investigated using the maximal aerobic test in a mechanically braked cycle ergometer (Cefise, Campinas, SP) with an initial load of 100 W and increments of 45 W x 2 min-1. The entire test was performed with a rotation of 90 rpm. During the test, heart rate (Polar RS800, Finland) and perceived exertion were evaluated using the Borg score scale [12]. The test was stopped when the participant was unable to maintain rotation at 90 rpm or voluntary withdrawal due to exhaustion. To determine the load to be used in the resistance protocol, the 12-repetition maximum (12-RM) test was performed, with a maximum of 5 attempts per apparatus (squat, leg press, lying leg curl and stiff exercises).

All protocols were performed in the morning. 24 hours before each exercise session, subjects did not exercise and did not consume alcohol and caffeine. One hour before the exercise, participants ate breakfast and repeated the same breakfast in all sessions. A standard snack was provided to volunteers after exercise.

Three exercise sessions were performed in a randomized crossover fashion design: continuous exercise (CE), high-intensity interval exercise (HIIE), and resistance exercise (RE). Sessions were separated by a minimum of 72 hours.

Continuous exercise (CE) protocol consisted of continuous cycling at 90 rpm for 60 min in a cycle ergometer at 50-60% of wVO_{2max}. High-intensity interval exercise (HIIE) protocol was carried out with 1-min cycling bouts at 100% of wVO_{2max}, interspersed with 1-min of passive recovery periods at 40% of VO_{2max} until voluntary exhaustion, at 90rpm. Resistance exercise (RE) protocol was performed with 3 sets of 12-repetition maximum

(12 RM) in the following sequence: squat (smith machine), leg press, lying leg curl and stiff exercises, with a 2-min recovery interval between sets and exercises. For the squat, a manual goniometer was used to determine the squat depth in 90° (±5°) knee angle. The feet were placed parallel, aligned with shoulders, and the malleolus was placed over a marker on the ground. The lowest point of the bar height for the squat depth was marked at the smith machine for each participant.

Saliva collection

Unstimulated saliva samples were collected in plastic tubes by spitting method, minutes after volunteers rinsed their mouth with water. All collection procedures were performed at the morning, at the following moments: at rest, immediately after the exercise session and 3 hours after. Samples were centrifuged at 3000g at 4°C for 20 minutes, and the supernatant was aliquoted. All samples were kept frozen at -80°C until analysis.

Spectra data evaluation

Measurements were performed in the Vertex 70 (Bruker Optics, Reinstetten, Germany) spectrophotometer using a micro-attenuated total reflectance (ATR) component, in mid-infrared region (3000–400 cm^{-1}) with spectral resolution of 4 cm^{-1} and 32 scans per spectrum. Samples were placed into ATR Diamond crystal with standardized pressure, and dried using airflow for 2 minutes. For the generation of mean spectra and band areas, the spectra were normalized by vector and baseline corrected to avoid errors

during the sample preparations and spectra analysis. The spectra data obtained were processed using Opus 6.5 software (Bruker Optics, Reinstetten, Germany). All samples were measured in triplicate.

Statistical analyses were performed using GraphPad Prism program (GraphPad Prism version 7.0 for Windows; GraphPad Software, San Diego, CA, USA). Data normality was determined using the Shapiro-Wilk test and analyzed using a one-way ANOVA with repeated measures (ANOVA-RM) followed by Tukey's posttest. Pearson correlations were computed between salivary spectrum bands and previous published data from salivary exercise intensity biomarkers. Random Forest machine learning algorithm was used to classify pre-exercise and post-exercise using the Orange Opus software. All data are reported as mean ±SEM. Differences were considered significant when $p<0.05$.

Results and Discussion

Salivary average FTIR spectrum at pre-exercise (Pre-ex), post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex) are shown in figure 1.

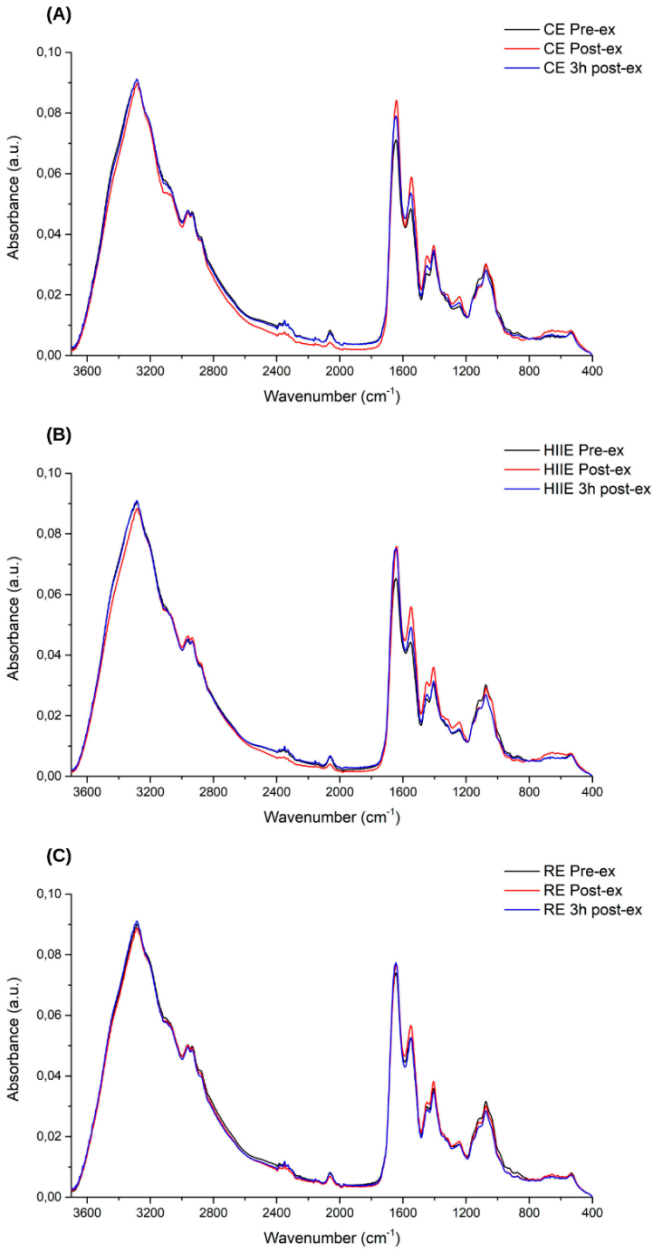

Figure 1. Salivary average FTIR spectrum at pre-exercise (Pre-ex), post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex). CE: continuous exercise (A); HIIE: high-

intensity interval exercise (B); and RE: resistance exercise (C). Black lines represents Pre-ex, Red lines Post-ex and Blue lines 3h post-ex.

The spectral analysis of saliva samples revealed 14 main peaks (Figures 2-4). Of these, 11 were differentially expressed, and the corresponding band assignments were described (Table 1 and 2, respectively).

The identified peaks of the salivary spectrum bands following continuous exercise (CE) are shown in figure 2. It was observed an increase of 2874 cm^{-1} band at post-exercise (post-ex) compared to pre-exercise (pre-ex), remaining increased 3h post-exercise (3h post) (Figure 2.C).

A decrease was verified of 2061 cm^{-1} band at post-ex compared to pre-ex. At 3 h post-ex, an increase was observed compared to post-ex (Figure 2.D).

The peak bands of 1640 cm^{-1} and 1549 cm^{-1} showed an increase at post-ex and 3h post-ex when compared to pre-ex. At 3 h post ex, both bands decreased compared to post-ex (Figures 2.E and 2.F, respectively).

Regarding 1449 cm^{-1} band, an increase was observed at post-ex compared to pre-ex (Figure 2.G).

It was also observed an increase in the bands 1317 cm^{-1}, 1241 cm^{-1} and 1159 cm^{-1} at post-ex compared to pre-ex. At 3 h post ex, these bands decreased when compared to that at post-ex (Figures 2.J, 2.K and 2.L, respectively).

No differences of 2962 cm^{-1}, 2935 cm^{-1}, 1404 cm^{-1}, 1340 cm^{-1},1118 cm^{-1}, and 1075 cm^{-1} bands were verified in CE (Figures 2.A, 2.B, 2.H, 2.I, 2.M, and 2.N).

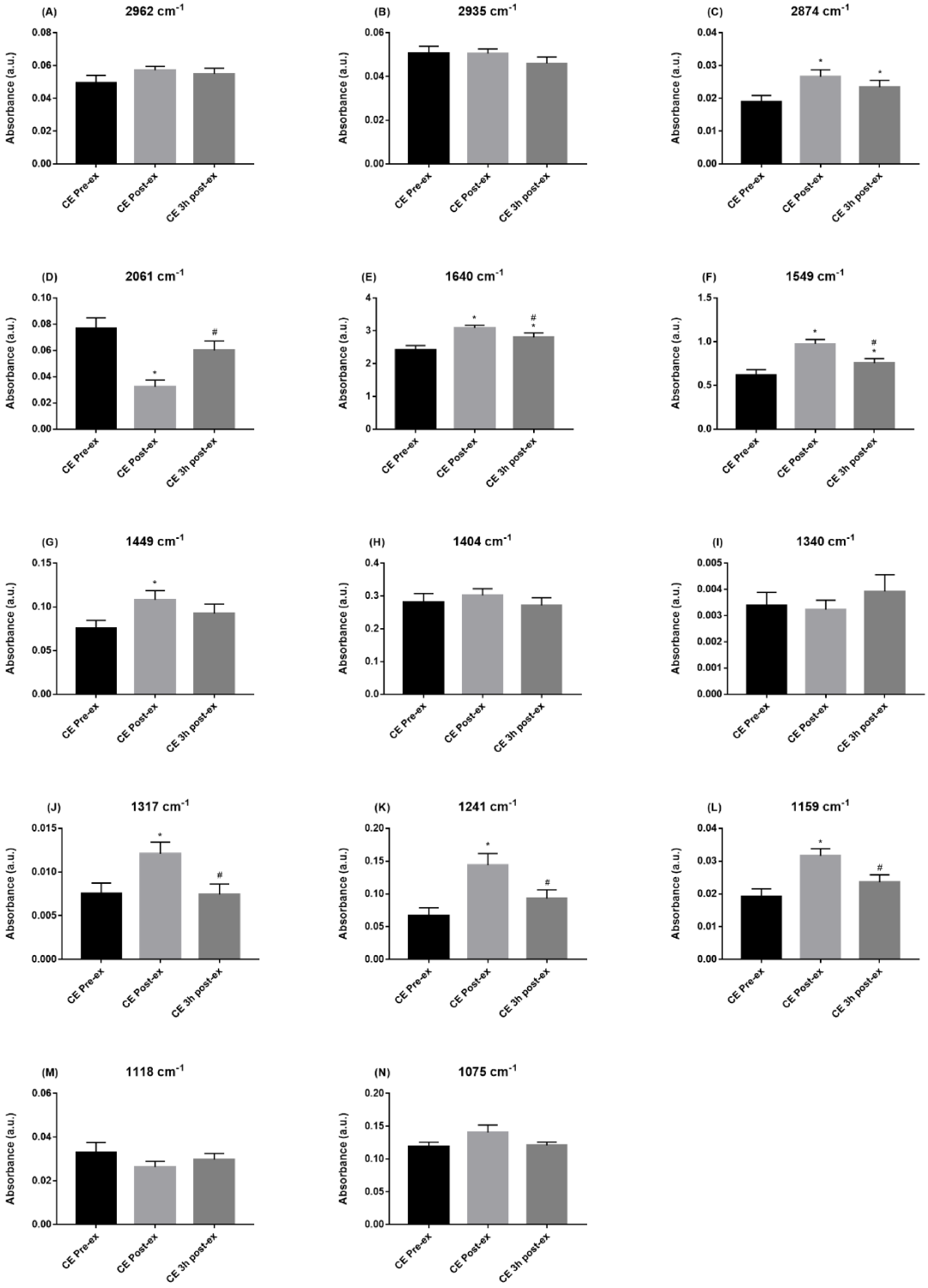

Figure 2. Bands of interest following continuous exercise (CE) at pre-exercise (Pre-ex), post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex). Identified band peaks: 2962 cm^{-1} (A); 2935 cm^{-1} (B); 2874 cm^{-1} (C); 2061 cm^{-1} (D); 1640 cm^{-1} (E); 1549 cm^{-1} (F); 1449 cm^{-1} (G); 1404 cm^{-1} (H); 1340 cm^{-1} (I); 1317 cm^{-1} (J); 1241 cm^{-1} (K); 1159 cm^{-}

^{1} (L); 1118 cm^{-1} (M); 1075 cm^{-1} (N). Values expressed as mean ± SEM. ∗p ≤ 0.05 vs. pre-ex; #p ≤ 0.05 vs. post-ex. ANOVA-RM followed by Tukey's test (n = 13).

HIIE also showed an increase of 2874 cm^{-1} band at post-ex compared to pre-ex, remaining increased at 3h after exercise (Figure 3.C).

In the same way as CE, a decrease was observed of 2061 cm^{-1} band at post-ex compared to pre-ex. Also, 3 h post-ex an increase was verified compared to post-ex (Figure 3.D).

1640 cm^{-1} and 1340 cm^{-1} bands increased 3 h post-ex when compared to pre-ex (Figures 3.E and 3.I, respectively).

An increase was verified of bands 1549 cm^{-1}, 1404 cm^{-1}, and 1159 cm^{-1} at post-ex when compared to pre-ex (Figures 3.F, 3.H and 3.L, respectively). While a decrease of 1118 cm^{-1} band was verified at 3 h post ex compared to post-ex (Figure 3.M).

1317 cm^{-1} and 1241 cm^{-1} bands increased at post-ex compared to pre-ex. At 3 h post ex, these bands decreased when compared to post-ex (Figures 3.J and 3.K, respectively).

No differences of 2962 cm^{-1}, 2935 cm^{-1}, 1449 cm^{-1}, and 1075 cm^{-1} bands were verified in HIIE (Figures 3.A, 3.B, 3.G, and 3.N).

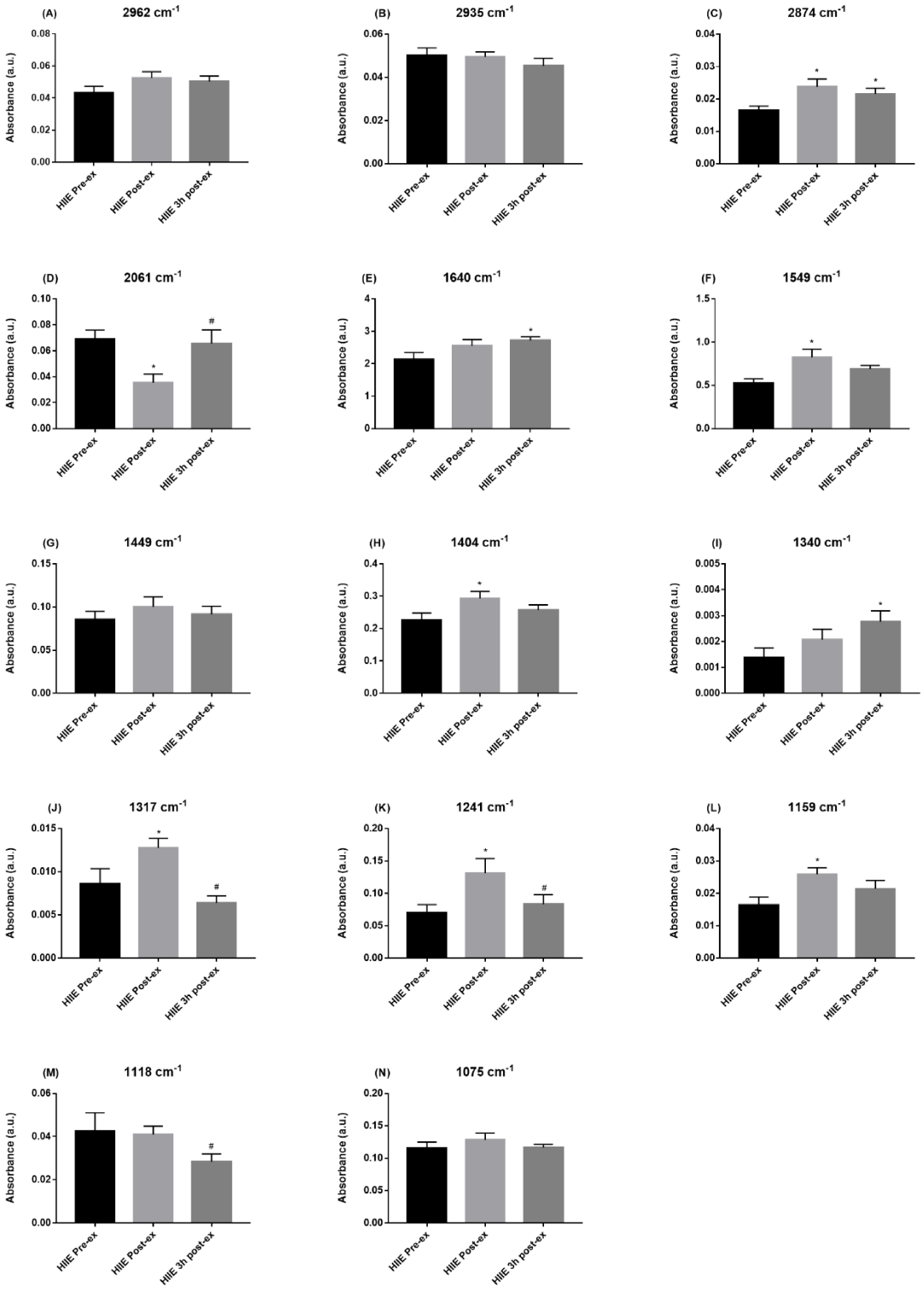

Figure 3. Bands of interest following high-intensity interval exercise (HIIE) at pre-exercise (Pre-ex), post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex). Identified band peaks: 2962 cm^{-1} (A); 2935 cm^{-1} (B); 2874 cm^{-1} (C); 2061 cm^{-1} (D); 1640 cm^{-1} (E); 1549 cm^{-1} (F); 1449 cm^{-1} (G); 1404 cm^{-1} (H); 1340 cm^{-1} (I); 1317 cm^{-1} (J); 1241 cm^{-1} (K); 1159 cm^{-1} (L); 1118 cm^{-1} (M); 1075 cm^{-1} (N). Values expressed as mean ±

SEM. $*p \leq 0.05$ vs. pre-ex; $\#p \leq 0.05$ vs. post-ex. ANOVA-RM followed by Tukey's test (n = 13).

The identified peaks of the salivary spectrum bands following resistance exercise (RE) are shown in figure 4. An increase of 1549 cm^{-1} and 1404 cm^{-1} bands were observed at post-ex compared to pre-ex (Figures 4.F and 4.H, respectively).

1241 cm^{-1} band increased at post-ex compared to pre-ex, remaining increased at 3h after exercise (Figure 4.K).

An increase of 1118 cm^{-1} band was observed at post-ex compared to pre-ex. At 3 h post ex, this band decreased when compared to post-ex (Figures 4.M).

No differences of 2962 cm^{-1}, 2935 cm^{-1}, 2874 cm^{-1}, 2061 cm^{-1}, 1640 cm^{-1},1449 cm^{-1}, 1340 cm^{-1},1317 cm^{-1}, 1159 cm^{-1}, and 1075 cm^{-1} bands were verified in RE (Figure 4.A, 4.B, 4.C, 4.D, 4.E, 4.G, 4.I, 4.J, 4.L and 4.N).

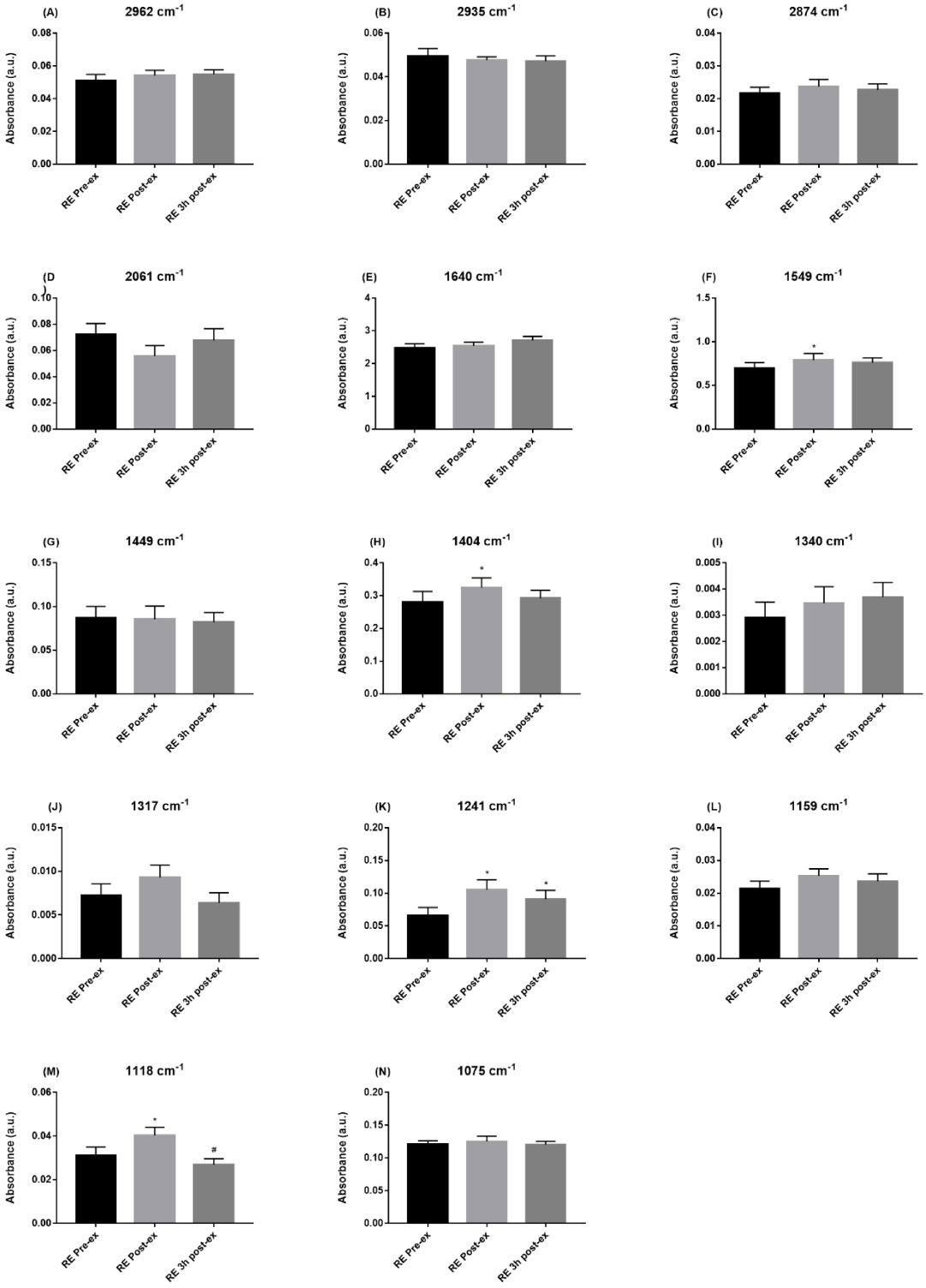

Figure 4. Bands of interest following resistance exercise (RE) at pre-exercise (Pre-ex), post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex). Identified band peaks: 2962 cm^{-1} (A); 2935 cm^{-1} (B); 2874 cm^{-1} (C); 2061 cm^{-1} (D); 1640 cm^{-1} (E); 1549 cm^{-1}

(F); 1449 cm^{-1} (G); 1404 cm^{-1} (H); 1340 cm^{-1} (I); 1317 cm^{-1} (J); 1241 cm^{-1} (K); 1159 cm^{-1} (L); 1118 cm^{-1} (M); 1075 cm^{-1} (N). values expressed as mean ± SEM. ∗p ≤ 0.05 vs. pre-ex; #p ≤ 0.05 vs. post-ex. ANOVA-RM followed by Tukey's test (n = 13).

An overview of the obtained results is shown in Table 2.

Table 2: Overview of the salivary spectral bands changes following the different exercise protocols,

	CE		HIIE		RE	
Wavenumber (cm^{-1})	**Post-ex**	**3h post-ex**	**Post-ex**	**3h post-ex**	**Post-ex**	**3h post-ex**
2874	↑*	↑*	↑*	↑*	-	-
2061	↓*	↑#	↓*	↑#	-	-
1640	↑*	↑* ↓#	-	↑*	-	-
1549	↑*	↑* ↓#	↑*	-	↑*	-
1449	↑*	-	-	-	-	-
1404	-	-	↑*	-	↑*	-
1340	-	-	-	↑*	-	-
1317	↑*	↓#	↑*	↓#	-	-
1241	↑*	↓#	↑*	↓#	↑*	↑*
1159	↑*	↓#	↑*	-	-	-
1118	-	-	-	↓#	↑*	↓#

Continuous exercise (CE), high-intensity interval exercise (HIIE) and resistance exercise (RE) at post-exercise (Post-ex), and 3 hours post-exercise (3h post-ex). ∗p ≤ 0.05 vs. pre-ex; #p ≤ 0.05 vs. post-ex.

The assignments of the different bands of interest are shown in Table 3. Significant differences were found in: symmetric stretching vibration of CH_3 of acyl chains (lipids) (2874 cm^{-1}), C-N stretching of thiocyanate anions (SCN^-) (2061 cm^{-1}), amide I band of proteins (1640 cm^{-1}), amide II band of proteins (1549 cm^{-1}), methyl groups of proteins (1449 cm^{-1}), CH_3 asymmetric

deformation of proteins (1404 cm^{-1}), CH_2 wagging and collagen (1340 cm^{-1}), amide III band components of proteins and collagen 1317 cm^{-1}), PO_2- asymmetric (phosphate I) (1241 cm^{-1}), C-O of proteins and carbohydrates (1159 cm^{-1}) and components such carbohydrates (1118 cm^{-1}).

Table 3: The spectral interpretations of salivary components.

Wavenumber (cm^{-1})	Band assignment
2874	Symmetric stretching vibration of CH_3 of acyl chains (lipids)
2061	C-N stretching of thiocyanate anions (SCN^-)
1640	Amide I band of protein
1549	Amide II band of proteins
1449	Asymmetric CH_3 bending modes of the methyl groups of proteins; Vibrations of CH_2 and/or CH_3 groups
1404	CH_3 asymmetric deformation of proteins
1340	CH_2 wagging and Collagen
1317	Amide III band components of proteins and Collagen
1241	PO_2^- asymmetric (phosphate I)
1159	C-O of proteins and carbohydrates
1118	C-O stretching vibration of C-OH group of ribose (RNA) Symmetric stretching P-O-C C-O stretching mode Mannose-6-phosphate Phosphorylated saccharide residue

In general, it is observed that many peaks increase right after exercise. The only band showing a post-exercise decline is the 2061 cm^{-1}, that exhibits the same pattern in CE and HIIE (decreasing post-ex and increasing 3 hours post-ex). This peak is assigned in the literature as thiocyanates (SCN^-) [13].

The thiocyanates are present in saliva as a free or loosely bound ion and has been studied mainly as a chemical indicator of cigarette smoking, being less explored in physical exercise [14,15]. A recent study by Mori and collaborators (2021), showed that the SCN^- concentrations, measured by capillary electrophoresis, decreased in the saliva samples of long-distance runners and sedentary students, after a short and a long run session, respectively [16]. This corroborates our data, and we therefore suggest the spectrum measurement of these thiocyanate ions (assigned as 2061 cm^{-1}) as potential markers of endurance exercise. Moreover, our data highlights the possibility of detecting thiocyanates in saliva through a non-invasive technique such as FTIR, which is important since the analysis of these ions can provide vital information on individual health [17].

The bands 1549 cm^{-1} and 1241 cm^{-1} increased after all types of exercises, suggesting them as possible biomarkers of exercise in general. The 1549 cm^{-1} spectrum band are commonly found in biological samples, and represents the vibration of protein amides I and II [18,19], while the 1241 cm^{-1} peak is mostly dominated by asymmetric vibrations of PO_2^- [20].

Interestingly, the 1449 cm^{-1} peak, that is related to asymmetric CH_3 bending modes of the methyl groups of proteins [21] was the only one that increased exclusively after CE. While the 1118 cm^{-1} peak, assigned as carbohydrates, increased exclusively in RE.

Vieira and collaborators (2021) verified a gradually increase of the 1404 cm^{-1} band (representative of methyl groups of proteins), with a progressive maximal exercise test, corroborating our data, in which this peak increased after HIIE and RE [22].

The spectrum peak of 1075 cm^{-1} (corresponding to phosphate/phospholipid molecules) was identified in the saliva samples; however, it did not show statistical differences comparing pre-ex, post-ex

and 3h post ex in the evaluated exercise protocols. This may be due to the high level of variation that this band presents during the practice of exercises. A recent study showed that this frequency range exhibit constant increases and decreases during the execution of a maximum progressive test in athletes [22].

Only few studies have evaluated salivary changes with exercise using FTIR [18,22-24]. Furthermore, they evaluated only one exercise protocol and one of them showed small sample size [22]. To our knowledge, this is the first study that compared changes in the saliva spectra following different types of exercises.

It is remarkable that CE showed a similar salivary spectrum pattern compared to HIIE, observed by the increase of the peaks 2874 cm^{-1}, 1549 cm^{-1}, 1317 cm^{-1},1241 cm^{-1} and 1159 cm^{-1} e decrease of the peak 2061 cm^{-1}. In our previous study, both protocols also showed similar responses regarding the oxidative stress response [8]. This is interesting, because the HIIE comprises a type of physical exercise that is performed in a much shorter interval time compared to the long-term endurance exercise. We attribute these similar spectral responses to the fact that HIIE presents characteristics related both to aerobic and anaerobic fitness, and shows increased metabolic fluctuations, which leads to activation of different signaling pathways [25,26].

In view of the large number of changes that have been promoted in the spectrum by these two types of exercise, we investigated whether the peaks altered by them could be related to salivary exercise intensity markers previously measured in these protocols [8]. For this, we analyzed the correlations of the peaks with the levels of total protein and salivary alpha-amylase activity.

In CE, it was observed a positive Pearson correlation of salivary total protein with the peaks 2874 cm^{-1} (pre-ex: r = 0.72; post-ex: r = 0.50; 3h post-ex: r= 0.72) and 1317 cm^{-1} (pre-ex: r = 0.81; post-ex: r = 0.52; 3h post-ex: r=0.71). Salivary amylase activity showed correlation with the peak 1640 cm^{-1} (pre-ex: r =0.62; post-ex: r = -0.18; 3h post-ex: r= 0.61). In HIIE, it was observed a positive Pearson correlation of the peak 2874 cm^{-1} with the salivary total protein (pre-ex: r = 0.80; post-ex: r = 0.66; 3h post-ex: r= 0.42) and the salivary amylase activity (pre-ex: r = 0.70; post-ex: r = 0.78; 3h post-ex: r= 0.39). These peaks represent lipids, amide I and III [13].

In order to determine whether these peaks that were correlated with the salivary exercise intensity markers can actually discriminate intensity through an algorithm, we have used machine learning analysis. For this, the normalized data of the peaks (2874 cm^{-1},1645-1639 $cm^{-1}$and 1317 cm^{-1}) were processed in the Orange Opus software and tested for different algorithms. As a result, we observed that the Random Forest machine learning technique showed the ability to discriminate before and after exercise, with a sensitivity of 61%, specificity of 69% and accuracy of 0.65. This test showed a good specificity for this discrimination, considering that there are no machine learning tests applied to saliva and FTIR in the literature. To our knowledge, this is the first study that developed an algorithm aimed to discriminate the intensity of physical exercise. With a larger sample in future studies, this algorithm may present higher specificity, sensitivity and accuracy.

Conclusion

This is the first study that compared changes in salivary spectra after different exercise protocols, suggesting spectrum peaks as biomarkers and the first to develop an algorithm that determines exercise intensity through a spectrum range. It was verified that the salivary biochemical components

changed differently according to the exercise protocol performed (resistance, continuous and high-intensity interval exercise), highlighting the peaks assigned as thiocyanates, amides, phosphates, asymmetric CH_3 bending modes of the methyl groups of proteins and carbohydrates.

Therefore, this study suggests ATR-FTIR as an efficient tool to evaluate metabolic changes promoted by exercise, allowing the use of saliva in a cost-effective manner for the study of athlete training state and performance.

Disclosure statement

The authors declare that there are no potential conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgments

The authors gratefully acknowledge the financial support of the National Council for Scientific and Technological Development (CNPq) and the Foundation for Research Support of the State of Minas Gerais (FAPEMIG). AVS, DDV, and DCC received graduate fellowships from the Coordination for the Improvement of Higher Education Personnel (CAPES) and CNPq. FSE is a grant recipient of CNPq.

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4.2 Artigo 2 –Título: *"Circulating Plasma MicroRNA Following Different Exercise Protocols: Increase of miR-23a With Continuous Endurance Exercise"*

Parecer do CEP consubstanciado (ANEXO A)

Normas de submissão para autores – Frontiers in Genetics (ANEXO C)

Circulating Plasma MicroRNA Following Different Exercise Protocols: Increase of miR-23a With Continuous Endurance Exercise

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Abstract

MicroRNAs (miRNAs) are small non-coding RNA molecules involved in the post-transcriptional regulation of gene expression. Plasma levels of circulating miRNAs change in response to acute exercise and training, so that these molecules are classified as mediators and are proposed as novel biomarkers of adaptive responses to exercise. However, it is not clear how their levels change according to type, duration and intensity of exercise. Thus, this work aimed to verify the acute effects of different exercise protocols on a panel of selected miRNAs previously related to important physiological modulations promoted by exercise. Literature analysis were used to select the miRNAs miR-133a, miR-126a, miR-146a, miR-221 and miR-23a. Healthy male volunteers (n=10) performed continuous exercise (CE), high-intensity interval exercise (HIIE), and resistance exercise (RE) protocols. RT-qPCR was used to determine the miRNA levels in plasma samples obtained at rest, immediately after the exercise session, and 3 hours after. To evaluate the possible targets of the altered miRNA expressed with exercise, the *in silico* miRDB tool was used. As result**,** it was observed an increase in miR-23a levels with CE, suggesting its effects on mitochondrial biogenesis pathways, since it targets the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Studies of this miRNA are limited, most of them analyze its expression within muscle. To our knowledge, the present work is the first to evaluate the expression of miR-23a in plasma samples following endurance exercise, contributing to a better understanding of this type of exercise with circulating miRNAs and possible effects over mitochondrial pathways.

Keywords: Mitochondria, Exercise protocols, Exercise epigenetics, Circulating miRNAs.

Introduction

The practice of physical exercise can increase life expectancy and lead to reduced risks of conditions such as cardiovascular disease, diabetes, hypertension and cancer. Exercise modalities can lead to health benefits through different ways. For example, resistance exercise shows a remarkable effect on increased muscle mass and strength, in addition to and enhancement of insulin sensitivity and glucose oxidation. Whereas aerobic exercise demonstrates pronounced effect on improvement of cardiorespiratory fitness, the levels of high-density lipoprotein cholesterol, triglycerides, fasting glucose and blood pressure (HAWLEY, 2004; PINCKARD; BASKIN; STANFORD, 2019; WARBURTON; NICOL; BREDIN, 2006; WEN; WAI; TSAI; YANG *et al.*, 2011).

Recent evidences suggests that high-intensity interval exercise, a burst-and-recover cycle, may be a viable alternative to the traditional aerobic endurance training, since it can be comparable or even more efficacious than continuous exercise for improving different parameters related to aerobic and anaerobic fitness, in addition to considerable improvements regarding the vascular system (GIBALA; LITTLE; MACDONALD; HAWLEY, 2012; RAMÍREZ-VÉLEZ; HERNÁNDEZ-QUIÑONES; TORDECILLA-SANDERS; ÁLVAREZ *et al.*, 2019; ZIEMANN; GRZYWACZ; LUSZCZYK; LASKOWSKI *et al.*, 2011).

Studies have shown that several processes inherent to exercise adaptation, such as angiogenesis, inflammation, mitochondrial metabolism and skeletal muscle hypertrophy can be regulated through microRNAs (miRNAs) (CHAN; ZHANG; HEMANN; MAHONEY *et al.*, 2009; DAVIDSEN; GALLAGHER; HARTMAN; TARNOPOLSKY *et al.*, 2011; DAVIDSON-

MONCADA; PAPAVASILIOU; TAM, 2010; FERNANDES; BARAUNA; NEGRAO; PHILLIPS *et al.*, 2015). This molecule consists of small non-coding RNA (usually 19–24 nucleotide in length) that plays important role in the post-transcriptional regulation of gene expression, by promoting the mRNA degradation or translational inhibition of proteins. Under physiological stimuli such as exercise or pathological process, these molecules can be released from different types of cells and tissues into the circulation, via vesicles or bound to proteins. These circulating miRNAs may then be transported to target cells and upregulate or downregulate important genes involved in different physiological processes (CONDRAT; THOMPSON; BARBU; BUGNAR *et al.*, 2020; ETHERIDGE; LEE; HOOD; GALAS *et al.*, 2011; MOHR; MOTT, 2015).

It is known that the expression of certain miRNAs can be altered according to the training status, the type and the intensity of the performed exercise (FARALDI; GOMARASCA; SANSONI; PEREGO *et al.*, 2019; FERNÁNDEZ-SANJURJO; ÚBEDA; FERNÁNDEZ-GARCÍA; DEL VALLE *et al.*, 2020; RAMOS; LO; ESTEPHAN; TAI *et al.*, 2018; UHLEMANN; MÖBIUS-WINKLER; FIKENZER; ADAM *et al.*, 2014). However, it is not clear how their levels actually change according to specific types of interventions. This knowledge of how different exercises can alter gene expression is fundamental to understanding the molecular processes that can lead to the adaptative effects of each protocol, as well as can lead to the establishment of new guidelines for the use of plasma in the diagnosis of physiological adaptation processes.

Thus, the aim of this study was to compare selected circulating miRNA levels, previously related to important physiological modulations promoted by exercise (miR-133a, miR-126a, miR-146a, miR-221 and miR-23a), in response

Material and methods

Subjects

This study was performed with 10 trained healthy males, who were nonsmoking, not taking regular/incidental medication or performance enhancing drugs. Subject characteristics are shown in Table 1.

Volunteers reported to be trained in both aerobic and anaerobic modalities, and to perform these exercises at least 3 times a week in the last 6 months.

All experimental procedures were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Institutional Review Board of the Federal University of Uberlandia (no. 1.908.151). The subjects were given informational briefings, and they provided voluntary, written informed consent for participation.

Table 1. Subject Characteristics.

Age (years)	*27±1.56*
Height (cm)	*175.6±1.67*
Weight (Kg)	*79.27 ±2.92*
BMI (Kg/m^2)	*25.68± 0.82*
Body Fat (%)	*18.02±1.68*

BMI: Body Mass Index. Values presented as means ± SEM, n = 10.

Experimental Procedures

Maximal Aerobic Capacity Test

The evaluation of the aerobic capacity of the subjects was performed using the maximal aerobic test in a mechanically braked cycle ergometer (Cefise, Campinas, SP) with an initial load of 100 W and an increment of 45 W every 2 minutes. The entire test was performed with a rotation of 90 rpm. During the test, heart rate (Polar RS800, Finland) and perceived exertion were evaluated using the Borg score scale (BORG, 1974). The criterion of interruption of the test was a perceived exertion scale score of 20 associated with inability to maintain rotation at 90 rpm and voluntary withdrawal due to exhaustion. The intensity associated with VO_{2max} (wVO_{2max}) was considered the last complete stage of the incremental test and this data was used to establish the loads of the high-intensity interval exercise (HIIE) and the continuous exercise (CE).

12-Repetition Maximum (12-RM) Test

The 12-repetition maximum (12-RM) test was performed to obtain the maximum load used in the resistance exercise (RE) protocol. Subjects performed the following exercises: squat (smith machine), leg press 45°, lying leg curl, and stiff. A maximum of 5 attempts per apparatus were performed, and the maximum load of each exercise was determined in the same sequence as the exercises performed in the acute experimental session.

Exercise Sessions

Subjects performed in a randomized crossover fashion design three exercise sessions on separate occasions, separated by a minimum of 72 hours:

continuous exercise (CE), high-intensity interval exercise (HIIE), and resistance exercise (RE).

The continuous exercise (CE) protocol was performed in a cycle ergometer (Cefise, Campinas, SP), and consisted of continuous cycling for 60 min at 50-60% of wVO_{2max}.

The high-intensity interval exercise (HIIE) protocol was carried out with 1 min cycling bouts at 100% of wVO_{2max}, interspersed with 1 min of passive recovery periods at 40% of VO_{2max} until voluntary exhaustion. Both aerobic exercises were performed with a rotation of 90 rpm.

The resistance exercise (RE) protocol was performed with 3 sets of 12-repetition maximum (12-RM) in squat (smith machine), leg press 45°, lying leg curl, and stiff exercises, in that order, with a 2 min recovery interval between sets and exercises. For the squat, feet were placed parallel, aligned with shoulders, and the malleolus was placed over a marker on the ground. A manual goniometer was used to determine the squat depth in 90° (±5°) knee angle. The lowest point of the bar height for the squat depth was marked at the smith machine for each participant.

The participants were instructed to follow normal diet and to avoid physical activity and refrain from consuming alcohol and caffeinated beverages for 24 h prior to each exercise session. Volunteers were also instructed to have breakfast one hour before the exercise session and to maintain the same diet for the breakfast in the subsequent experimental sessions. A standard snack was provided to the subjects just after exercise. After that, the subjects stayed in fasting condition until the last collection. All protocols were performed in the

morning and all exercises were guided, monitored and properly explained by professional physical educators.

Sample Collection

Blood from the antecubital vein was collected from the subjects and placed in EDTA-coated tubes by a qualified phlebotomist using standardized venipuncture techniques. All collection procedures were performed in the morning, at the following moments: at rest, immediately after the exercise session, and 3 hours after. These time points were selected in order to verify the effects promoted right after exercise and also to evaluate the recovery time of these miRNAs.

Blood samples were centrifuged at 3000 rpm at 4°C for 20 minutes, and the supernatant was aliquoted. All plasma samples were flash-frozen in liquid nitrogen, and kept frozen at -80°C until analysis.

Sample quality analysis

After a first visual inspection for pink/red discoloration (indicative of hemolyzed samples), the level of hemolysis in plasma samples was assessed spectrophotometrically measuring the absorbance of hemoglobin at 414 nm (ND-1000, NanoDrop™, Thermo Fisher Scientific, Wilmington, DE). Plasma samples with absorbance values greater than 0.3 were considered hemolyzed (SHAH; SOON; MARSH, 2016).

Analysis of circulating miRNAs

Total RNA enriched for miRNAs was extracted from 100uL of plasma samples using MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems, USA), using magnetic-bead technology that enables reproducible recovery of high-quality RNA. All procedures were performed according to the manufacturer's protocol. The synthetic *Caenorhabditis elegans* miRNA Cel-miR-39-3p was used as an exogenous control (spike-in) to ensure the reproducible and accurate quantification of circulating miRNA levels.

Quantification of samples concentration and purity was carried out by measuring optical density using Nanodrop (ND-1000, NanoDrop™, Thermo Fisher Scientific, Wilmington, DE).

The cDNA was synthesized using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, USA) also according to the user guidelines. This kit uses universal primers that uniformly amplify the amount of cDNA for each target, increasing the assay sensitivity.

For quantitative real-time PCR (qRT-PCR), the TaqMan® Advanced miRNA Assays was used for quantification of 5 miRNAs selected based on involvement in pathways relevant to physical exercise adaptation: miR-133a, miR-126, miR-146a, miR-221, miR-23a, and the control cel-miR-39. Characteristics of these miRNAs are shown in Table 2.

Table 2. Characteristics of the evaluated miRNAs.

miRBase ID	Chromosome Location	Mature miRNA Sequence	Assay ID
hsa-miR-133a-3p	Chr.18: 21825698 - 21825785 [-] on Build GRCh38	UUUGGUCCCCUUCAACCAGCUG	478511_mir
hsa-miR-126-3p	Chr.9: 136670602 - 136670686 [+] on Build GRCh38	UCGUACCGUGAGUAAUAAUGCG	477887_mir
hsa-miR-146a-5p	Chr.5: 160485352 - 160485450 [+] on Build GRCh38	UGAGAACUGAAUUCCAUGGGUU	478399_mir
hsa-miR-221-3p	Chr.X: 45746157 - 45746266 [-] on Build GRCh38	AGCUACAUUGUCUGCUGGGUUUC	477981_mir
hsa-miR-23a-3p	Chr.19: 13836587 - 13836659 [-] on Build GRCh38	AUCACAUUGCCAGGGAUUUCC	478532_mir
cel-miR-39-3p	-	UCACCGGGUGUAAAUCAGCUUG	478293_mir

The 7300 Real Time PCR Systems apparatus (Applied Biosystems) was used. Cycle conditions were: 50 °C for 2 min, 95°C for 10 min followed by 50

cycles at 95 °C for 15 s and 60 °C for 60 s. Analyses were performed from the cycle threshold (Ct) using DataAssist v3.01 software (Applied Biosystems). PCR was carried out in duplicate for each sample. The relative levels of miRNA in subject samples were presented as fold change using the $2^{-\Delta\Delta Ct}$ formula.

miRNA target prediction

To evaluate the possible targets of the altered miRNA expressed with exercise, the miRDB tool was used, an online database for prediction of functional microRNA targets (CHEN; WANG, 2020).

Statistical Analysis

Analyses were performed in the GraphPad Prism program (GraphPad Prism version 7.0 for Windows; GraphPad Software, San Diego, CA, USA). Data normality was determined using the Shapiro-Wilk test and analyzed using a one-way ANOVA with repeated measures (ANOVA-RM) followed by Dunn's posttest. All data are reported as mean ±SEM. Differences were considered significant when $p<0.05$.

Results and Discussion

Sample quality analysis

Given that blood cells could release microRNAs into plasma samples in the hemolysis process, this could interfere into microRNA plasma profile and therefore impair the use of specific microRNAs as a biomarker (SHAH; SOON; MARSH, 2016). Although many miRNAs do not change with hemolysis and can be used as biomarkers in these conditions, this is an important analysis to be made in order to avoid any systematic bias (BLONDAL; JENSBY NIELSEN; BAKER; ANDREASEN et al., 2013).

Shah and collaborators identified the absorbance cutoff of 0.3 at 414 nm as a predictor of samples with high levels of hemolysis, correlating with the data obtained by visual inspection and the miR ratio (SHAH; SOON; MARSH, 2016). According to this, the assessment of plasma samples in this study showed the low average percentage of hemolysis of 5.55%, what we believe does not interfere with the analyzed miRNA expression.

Plasmatic circulating miRNA expression

The expression data of microRNAs related to Continuous Exercise (CE) are shown in Figure 1. An increase in circulating miR-23a was observed when comparing rest with after exercise (p=0.02), while a decrease was verified 3 hours post-exercise (p=0,0005) (Figure 1E).

No differences were observed in the expressions of the circulating miR-133a (Figure 1A), miR-126 (Figure 1B), miR-146a (Figure 1C) and miR-221 (Figure 1D).

Regarding High-intensity interval exercise (HIIE), no significant alterations were verified in the expression of the evaluated miRNAs (miR-133a, miR-126, miR-146a, miR-221 and miR-23a) (Figures 2A, 2B, 2C, 2D and 2E). Likewise, no expression changes were observed in the Resistance Exercise (RE) protocol (Figure 3).

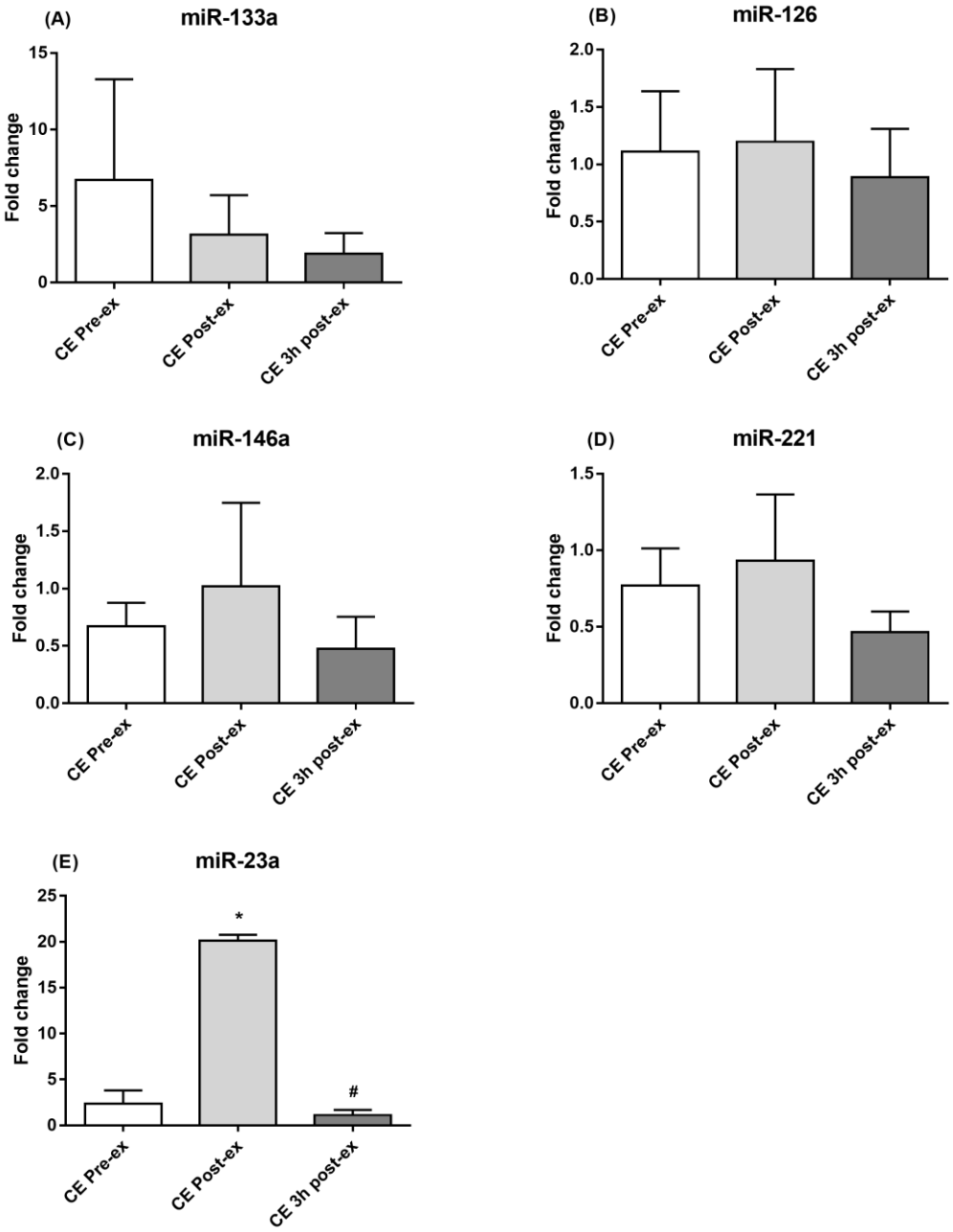

Figure 1: Continuous exercise (CE) plasmatic levels of miR-133a (A), miR-126 (B), miR-146a (C), miR-221 (D) and miR-23a (E) normalized by cel-miR-39, at pre-exercise, post-exercise, and 3 hours post-exercise. Values expressed as mean ±SEM. *$p<0.05$ vs. pre-ex; $^{\#}p<0.05$ vs. post-ex. ANOVA-RM followed by Dunn's test (n=10).

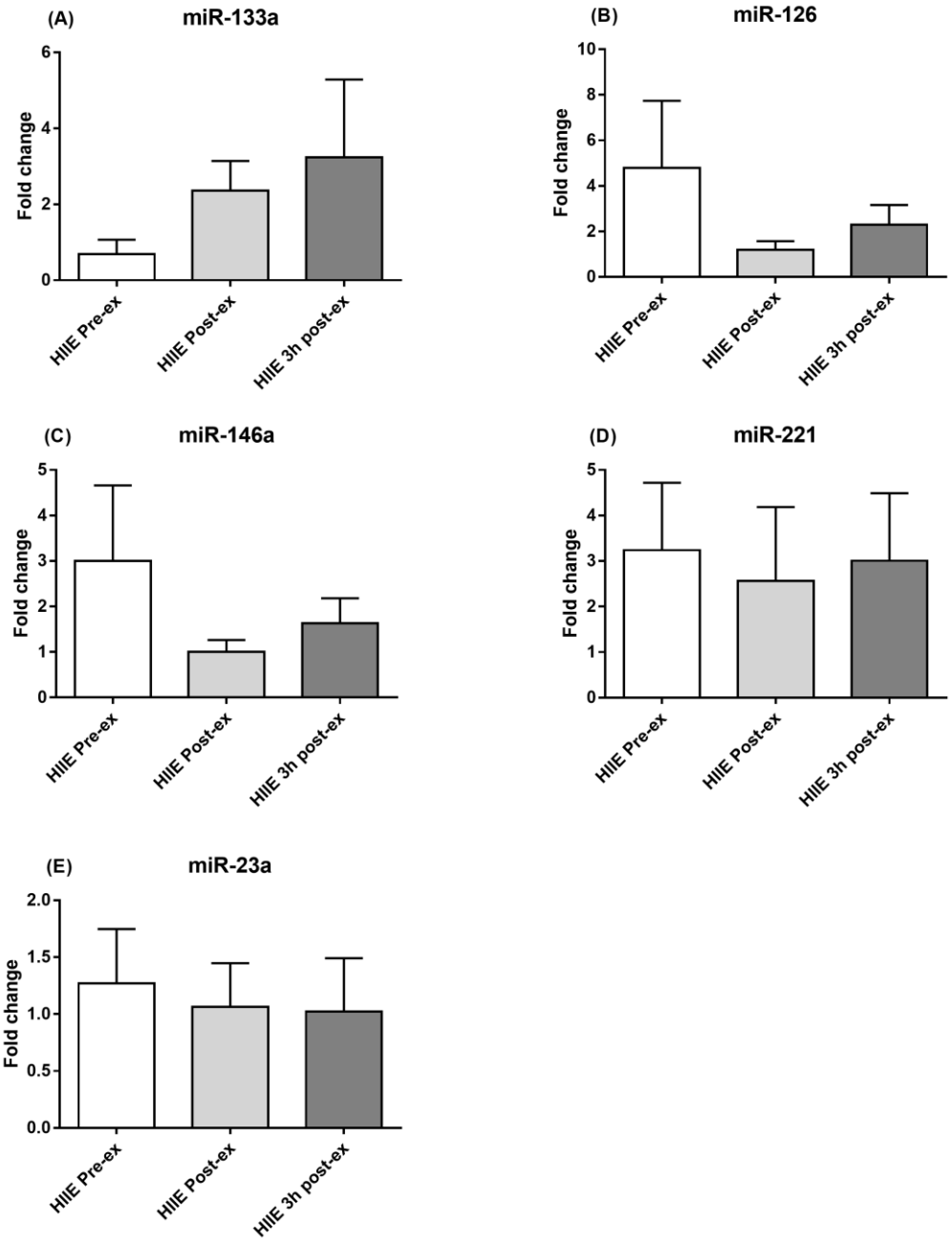

Figure 2: High-Intensity Interval Exercise (HIIE) plasmatic levels of miR-133a (A), miR-126 (B), miR-146a (C), miR-221 (D) and miR-23a (E) normalized by cel-miR-39, at pre-exercise, post-exercise, and 3 hours post-exercise. Values expressed as mean ±SEM. *$p<0.05$ vs. pre-ex; $^{\#}p<0.05$ vs. post-ex. ANOVA-RM followed by Dunn's test (n=10).

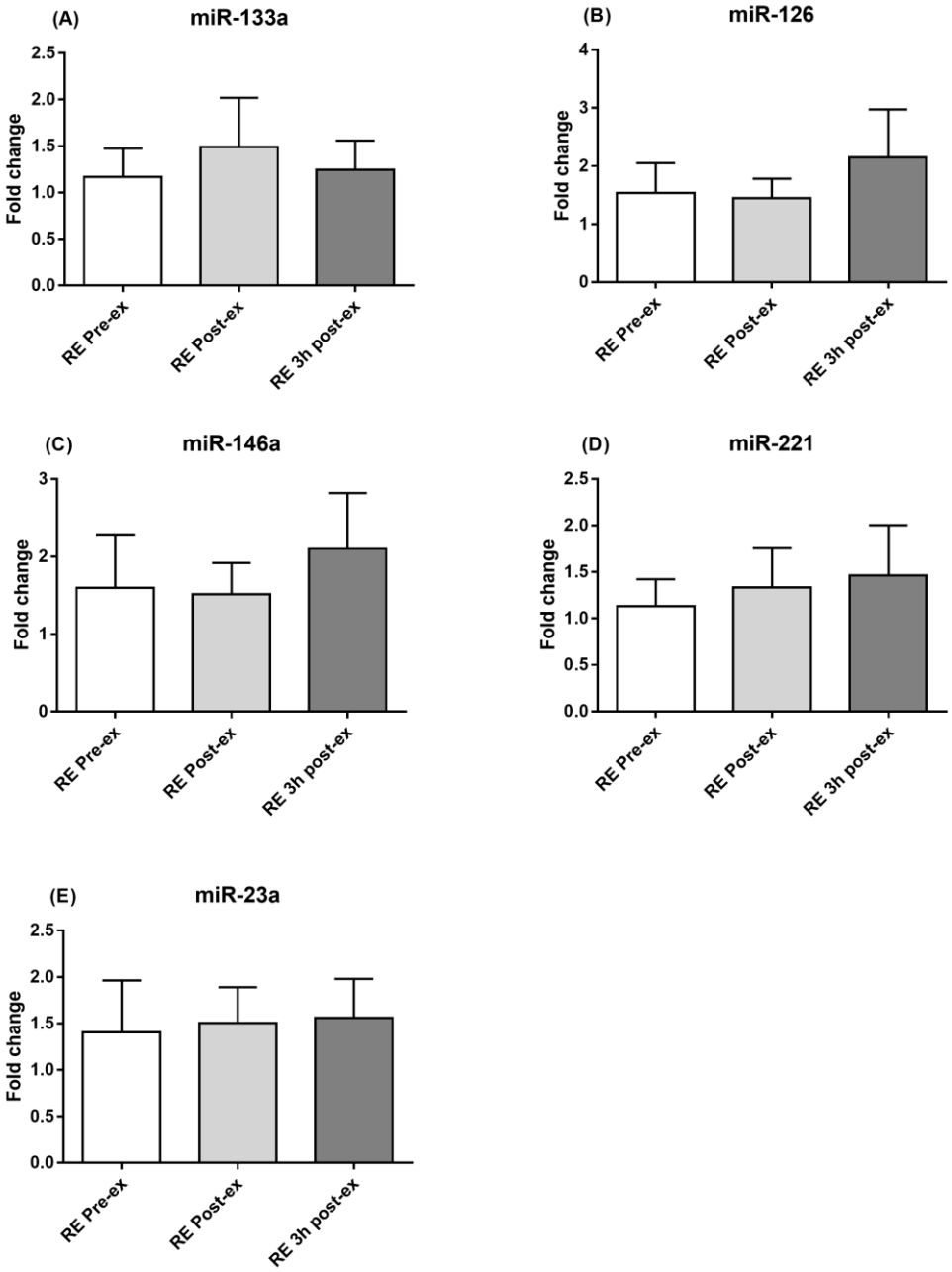

Figure 3: Resistance exercise (RE) plasmatic levels of miR-133a (A), miR-126 (B), miR-146a (C), miR-221 (D) and miR-23a (E) normalized by cel-miR-39, at pre-exercise, post-exercise, and 3 hours post-exercise. Values expressed as mean ±SEM. *$p<0.05$ vs. pre-ex; $^{\#}p<0.05$ vs. post-ex. ANOVA-RM followed by Dunn's test (n=10).

It was verified that a single acute session of aerobic continuous exercise was able to differentially alter the expression of the miR-23a, which is involved in the regulation of important physiological mechanisms of adaptation to exercise and other important physiological processes. The analyzes performed by the miRDB tool predicted 1457 targets for miR-23a, such as genes that encode for zinc finger proteins, semaphorin 6D, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), TGF-beta, different phosphatases, among several targets.

The PGC-1α shows a high relationship with the practice of endurance exercises. Other *in silico* analyzes using different databases (MiRANDA: 2005 build, MiRANDA: 2006 build, PicTAR, and TargetScan 2006 build) also showed the PGC-1α as an important potential target of miRNA-23a, therefore being directly related to the processes of mitochondrial biogenesis (JI; GOMEZ-CABRERA; VINA, 2006; LEWIS; BURGE; BARTEL, 2005).

Safdar and collaborators (2009), when evaluating the effect of endurance exercise on the expression of microRNAs in the muscle of C57Bl/6J mice, verified the reduced expression of miR-23a, which was accompanied by an increase in the expression of PGC-1 α, reaffirming the effects of this microRNA upon mitochondrial activity as a negative modulator (SAFDAR; ABADI; AKHTAR; HETTINGA *et al.*, 2009). Another study by Russel and colleagues (2013), also observed decreased levels of miR-23a after endurance exercise, this time evaluating human skeletal muscle (RUSSELL; LAMON; BOON; WADA *et al.*, 2013). These results disagree with the data obtained in the present work, in which there was an increase in the expression of this miRNA. However, it is important to highlight the fact that the analysis of circulatory miRNAs may not correspond to their responses within skeletal muscle (D'SOUZA;

MARKWORTH; AASEN; ZENG *et al.*, 2017; VAN PELT; VECHETTI; LAWRENCE; VAN PELT *et al.*, 2020).

A study which aimed to verify the relationship between serum miRNAs and muscle atrophy, showed that in rats there was little relationship between miR-23a-3p found in extracellular vesicles from serum and the miR-23a-3p content within the muscle, kidney, and liver (VAN PELT; VECHETTI; LAWRENCE; VAN PELT *et al.*, 2020).

Moreover, D'Souza and collaborators (2020) compared the expression of some circulatory and skeletal muscle miRNAs in young men after an acute session of resistance exercise, and observed that miR-23a levels decreased within muscle, whereas no changes were verified in the plasma samples. Interestingly, this lack of agreement was verified in several other miRNAs analyzed (D'SOUZA; MARKWORTH; AASEN; ZENG *et al.*, 2017). Taken together, these data draw attention to the importance of evaluating the source of the analyzed miRNA in order to better understand its behavior (increase/decrease) an to predict its function.

A study made with highly trained male athletes showed an increase of miR-23a levels in the whole blood leukocytes after a treadmill ramp test to exhaustion, which corroborate our data. In addition to mir-23a, other miRNAs belonging to the same cluster (mir-24, and mir-27a) were also elevated after the exercise (MAKAROVA; MALTSEVA; GALATENKO; ABBASI *et al.*, 2014).This cluster is involved in biological processes such as angiogenesis, haematopoiesis, cardiac hypertrophy and several immune-related pathways (CHHABRA; DUBEY; SAINI, 2010).

Studies analyzing this miRNA are limited. Here, we demonstrated the increase of miR-23a following continuous exercise in trained men. To our knowledge, the present work is the first to evaluate the expression of miR-23a in plasma samples following endurance exercise, modality that is closely related to mitochondrial biogenesis processes (important target of this miRNA) (IRRCHER; ADHIHETTY; JOSEPH; LJUBICIC *et al.*, 2003).

Since most of the physical exercise studies analyzed miRNA-23a within muscles, this work evaluating circulatory levels may contribute to a better understanding of the tissues cross-talking signaling processes promoted by exercise and mediated by plasma miRNAs. The miR-23a is not muscle-specific and seem to control important functions which muscle cells share with other cells.

The lack of significant differences in other miRNAs following CE and the miRNA levels following HIIE and RE were surprising. Specifically, we hypothesized that HIIE would show greater difference, once it is reported that this type of exercise may present comparable or even greater changes in many stimuli, such as related to oxidative stress, improved skeletal muscle metabolic control and changes in circulating factors (GIBALA; LITTLE; MACDONALD; HAWLEY, 2012; SOUZA; GIOLO; TEIXEIRA; VILELA *et al.*, 2019). In addition, a study by Cui and collaborators (2016) showed that HIIE exerts similar responses on miR-1, miR-133a, miR-133b and miR-206 levels, altering their expression in the same way as a continuous exercise (CUI; WANG; YIN; TIAN *et al.*, 2016).

There are few studies comparing the circulating miRNA profile following different types of exercise (CUI; SUN; YIN; GUO *et al.*, 2017; CUI; WANG; YIN; TIAN *et al.*, 2016; HORAK; ZLAMAL; ILIEV; KUCERA *et al.*, 2018;

SAPP; CHESNEY; EAGAN; EVANS *et al.*, 2020; SAPP; EVANS; EAGAN; CHESNEY *et al.*, 2019; UHLEMANN; MÖBIUS-WINKLER; FIKENZER; ADAM *et al.*, 2014). In a recent study Sapp and collaborators verified an increase of miR-21-5p, 126-3p, 126-5p, 150-5p, 155-5p, and 181b-5p after HIIE exercise while only miR-150-5p and 221-3p increased after moderated continuous exercise (SAPP; CHESNEY; EAGAN; EVANS *et al.*, 2020). It is interesting to mention that it was evaluated a shorter moderated bout compared to our study (30-min versus 60-min exercise).

A study by Uhlemann and collaborators comparing the plasma concentration of miRNA-126 and miRNA-133, verified that different endurance exercise protocols lead to an increase in miRNA-126 levels (indicating its effect on endothelial cells), while resistance exercise promoted changes in microRNA 133 (marker for muscle damage) (UHLEMANN; MÖBIUS-WINKLER; FIKENZER; ADAM *et al.*, 2014).

The contrasting results of our study compared to previous studies, or even among themselves, may stem from different factors such as different methodological approaches, normalization methods (use of exogenous or endogenous control), sample quality (many studies do not present quality measurements to assess their sampling protocol performance), the selection of the study population and sampling time. A limitation of our study consisted in the limited number of analyzed miRNAs that may limit our understanding of their regulatory role.

Conclusion

A single acute session of CE differentially altered the expression of miR-23a, whereas no changes were observed in HIIE and RE. To our knowledge, the present work is the first to evaluate the expression of miR-23a in plasma samples following endurance exercise. This contributes to a better understanding of the relation of this type of exercise over mitochondrial biogenesis pathways (important target of this miRNA) and this circulatory finding may help to elucidate the signaling tissues cross-talking processes mediated by this miRNA.

Disclosure statement

The authors declare that there are no potential conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgments

The authors gratefully acknowledge the financial support of the National Council for Scientific and Technological Development (CNPq) and the Foundation for Research Support of the State of Minas Gerais (FAPEMIG). AVS, DDV, and DCC received graduate fellowships from the Coordination for the Improvement of Higher Education Personnel (CAPES) and CNPq. FSE is a grant recipient of CNPq.

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ANEXO A – Parecer Consubstanciado CEP

UFU Comitê de Ética em Pesquisa — UNIVERSIDADE FEDERAL DE UBERLÂNDIA/MG — Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Biotecnologia em diagnóstico de microRNAs circulantes do plasma como potenciais biomarcadores do exercício e dos seus benefícios para saúde

Pesquisador: Foued Salmen Espindola
Área Temática:
Versão: 3
CAAE: 60070216.2.0000.5152
Instituição Proponente: Instituto de Genética e Bioquímica
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.908.151

ANEXO B - Normas de submissão para autores - Scientific Reports

https://www.nature.com/srep/author-instructions/submission-guidelines

ANEXO C – Normas de submissão para autores – Frontiers in Genetics

https://www.frontiersin.org/guidelines/author-guidelines